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ORIGINAL ARTICLE

Natural course and prognostic factors of chronic urticaria in Korean children: A single center experience

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Abstract

Background: Chronic urticaria (CU) has an adverse effect on academic achievement and psychosocial development in children.

Objective: We aimed to investigate the natural course of CU and to identify relevant factors associated with a poor CU prognosis in Korean children.

Methods: We retrospectively analyzed 253 children with episodes of wheals or angioedema at least 3 times a week that persisted for at least 6 weeks. Clinical data and laboratory results were obtained from medical records and parental telephone interviews. Kaplan-Meier survival analysis and log rank tests were performed to assess the median time to remission of CU and prognostic factors.

Results: Median age at onset was 5.0 years (interquartile range, 2.5-9.1) and median follow-up period was 7.6 months (interquartile range, 3.9-19.7). Of 253 patients, 68.8% had chronic inducible urticaria and 31.2% had chronic spontaneous urticaria. Physical urticaria was the only cause of chronic inducible urticaria, and the most common physical urticaria was dermographism. Median duration to remission of CU was 10.2 months (95% confidence intervals, 8.0-12.5 months). Kaplan-Meier analysis revealed that 33.4%, 53.0%, and 71.2% of children were in remission at 6, 12, and 24 months, respectively, after the onset of CU. The presence of allergic sensitization was significantly associated with a poor CU prognosis in univariable and multivariable analyses ($P = 0.010$ and $P = 0.014$, respectively).

Conclusion: Half of children with CU were in remission 10.2 months after onset. Allergic sensitization was a risk factor associated with longer duration CU.

Key words: Chronic urticaria; Etiology; Prognosis; Children; Korea

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Introduction

Chronic urticaria (CU) is defined as wheals with recurrent episodes lasting for at least 6 weeks. The prevalence of CU has been reported to be 0.5-5.0% in the general population and about 0.1-0.3% in children.[1] It is known that children have a lower prevalence than adults.[1] Impairment of quality of life in patients with CU is comparable to that experienced by patients with other chronic diseases such as ischemic heart disease, cystic fibrosis, and epilepsy.[2,3] CU impairs physical activity and lowers social function, which can adversely affect academic achievement and psychosocial development in children and adolescents.[3-5]

Acute urticaria is frequently associated with identifiable causes; in contrast, it is difficult to identify the etiology and to predict the occurrence or exacerbation of symptoms in patients with CU.[3,6] CU is classified into chronic spontaneous urticaria (CSU) and chronic inducible urticaria (CIU) according to

whether symptoms occur spontaneously or are induced by specific triggers. Although its pathogenesis is poorly understood, mixed Th1/Th2 immune response, mast cell activation, and autoimmunity are considered to play a role in the pathogenic mechanisms of CU.[7] There are also several types of evidence supporting that autoantigen is synthesized by specific triggers leading to mast cell degranulation in patients with CIU.[8,9] In general, the duration of CSU is 1-5 years, but is likely to be longer in more severe cases.[10] A previous Korean study reported that the mean duration of CU was 3.76 years in 641 adults.[11] The co-existence of angioedema, severity, and the presence of anti-thyroid antibodies were associated with longer disease duration in adult patients with CU.[12,13] However, few studies have investigated the clinical course or identified prognostic factors of CU in children.

Providing correct information about the etiology and prognosis of CU can reduce the anxiety of pediatric patients and their parents and help establish a therapeutic plan. Therefore, our aims in this study were to investigate the clinical features and natural course of CU and to identify factors associated with a poor CU prognosis in Korean children.

Methods

Patients

We retrospectively reviewed the medical records of patients 18 years or younger that were diagnosed with CU in Samsung Medical Center from January 2007 to October 2015. The definition of CU was wheals or angioedema that had lasted for more than 6 weeks and that occurred repeatedly (more than 3 times a week).

Demographic and clinical data were collected, including gender, age at onset, duration and frequency of symptoms, extent of skin involvement, presence of angioedema, and personal and familial history of allergic or autoimmune diseases. A family history of allergic diseases was defined as the presence of one or more family members diagnosed with atopic dermatitis, asthma, or allergic rhinitis by a physician.

All patients were interviewed using a detailed questionnaire regarding trigger factors such as sunlight, cold, water, pressure, vibration, exercise, scratching, foods, and drugs. Food or drug allergy was defined as a convincing history of reproducible symptoms within two hours after exposure to a single food or drug. Physical urticaria was diagnosed according to the criteria described by the Joint Task Force on Practice Parameters.[14] In addition, medication use for CU (H1 receptor antagonists, H2 receptor antagonists, leukotriene antagonists, oral corticosteroids, immunosuppressants, and biologic agents) was investigated.

For patients who did not have data regarding remission of clinical symptoms on their medical chart, a telephone survey was utilized based on a preformulated questionnaire. Complete remission of CU was defined as the absence of urticaria for at least 4 weeks without any medication.[13] This study was approved by the Institutional Review Board (IRB) of Samsung Medical Center (IRB No.2015-06-188).

Laboratory test

Eosinophils in peripheral blood, erythrocyte sedimentary rate (ESR), thyroid stimulating hormone (TSH), free thyroxine (T4), triiodothyronine (T3), thyroid auto-antibodies (thyroglobulin antibody, antimicrosomal antibody, and TSH receptor antibody), antinuclear antibody (ANA), serum C-reactive protein (CRP) levels, and serum total immunoglobulin E (IgE) levels were measured. The ANA test was performed by using the indirect immunofluorescence method with sera (Fluoro Hepana Test, MBL, Nagoya, Japan). Skin prick tests (Allergopharma, Reinbek, Germany) on the back or measurement of specific IgE levels with the immunoCAP system (Thermo Fisher Scientific Inc., Waltham, MA, USA) were performed regarding the following common allergens: *Dermatophagoides pteronyssinus*, *D. farinae*, egg white, cow's milk, wheat, soybean, and peanut. Positive sensitization was defined as a wheal diameter ≥ 3 mm or specific IgE levels ≥ 0.35 kU/L. Eosinophilia was defined as when eosinophils comprised more than 4% of total leukocytes.

Statistical analysis

Data for continuous variables were shown as median and interquartile range. Prevalence rates were shown as percentages. Cumulative survival curves were estimated by the Kaplan -Meier method, and relationships between the cumulative probability of complete remission of CU and prognostic factors were analyzed using a log-rank test. The influence of prognostic factors on remission was evaluated by univariable Cox regression analyses. The relative importance of multiple prognostic factors on CU remission was analyzed using multivariable analysis in conjunction with the Cox proportional regression model. Variables with a P value of less than 0.2 in univariable analysis were chosen for multivariable analysis. Candidate variables for adjustment included gender, age at onset (< 5 years old or ≥ 5 years old), clinical symptoms (with or without angioedema), past history of allergic diseases (presence or absence), past history of autoimmune diseases (presence or absence), family history of allergic diseases (presence or absence), inducible factors (presence or absence), sensitization (negative or positive), total IgE (< 200 kU/L or ≥ 200 kU/L), eosinophils (< 4% or ≥ 4%), ESR (< 20 mm/hr or ≥ 20 mm/hr), CRP (< 0.5 mg/dl or ≥ 0.5 mg/dl), and ANA (negative or positive). CRP and ANA were excluded from the multivariate analysis because data were available for only 80 and 88 cases, respectively. Statistical analyses were performed using SAS (version 9.3, SAS Institute, Inc., Cary, NC, USA). Statistical significance was defined as a P value < 0.05.

Results

A total of 253 patients (142 boys and 111 girls) with CU were included in this study. Median age at onset was 5.0 years (interquartile range, 2.5-9.1) and median follow-up period was 7.6 months (interquartile range, 3.9-19.7 months) (**Table 1**). Overall, 29 (11.5%) patients had wheals accompanied with angioedema, while 224 (88.5%) patients had wheals only without angioedema. Of 253 patients, 68.8% (174/253) had CIU, while 31.2% (79/253) had CSU. The only cause of 174 patients with CIU was physical urticaria, and the most common physical urticaria was dermographism (128/174, 73.6%), followed by cholinergic urticaria (76/174, 43.7%), cold urticaria (15/174,

Table 1. Subject characteristics and laboratory results (n = 253).

Characteristic	Number (%)
Gender	
Male	142 (56.1)
Female	111 (43.9)
Age (years)*	5.0 (2.5-9.1)
< 1	15 (5.9)
1-4	111 (43.9)
5-9	78 (30.8)
≥ 10	49 (19.4)
Clinical symptoms	
Wheals only	224 (88.5)
Angioedema with wheals	29 (11.5)
Past medical history	
Atopic dermatitis	52 (20.1)
Allergic rhinitis	38 (15.0)
Asthma	24 (9.5)
Autoimmune disease	7 (2.8)
Family history of allergic diseases	
Atopic dermatitis	20 (7.9)
Allergic rhinitis	38 (15.0)
Asthma	8 (3.2)
Allergic sensitization**	90/219 (41.1)
Log(total IgE, kU/L)	2.0 ± 0.5

*Data are presented as medians (interquartile range).
** 219 patients underwent either a skin prick test or ImmunoCAP test.

8.6%), delayed pressure urticaria (9/174, 5.2%), solar urticaria (3/174, 1.7%), and aquagenic urticaria (2/174, 1.1%).

Twenty-two (25.0%) of 88 patients who underwent testing for ANA were positive. However, only one of these 22 patients had a history of autoimmune diseases such as systemic lupus erythematosus. TSH was elevated in only one of the 50 patients (2.0%) who underwent a thyroid function test, but her T3 and free T4 levels were normal.

During the observation period, 195 (77.0%) children had complete remission. In Kaplan-Meier survival analysis, 33.4%, 53.0%, and 71.2% of patients recovered at 6, 12, and 24 months after symptom onset, respectively (**Figure 1**). The median duration of CU was 10.2 months (95% confidence intervals, 8.0-12.5 months). A total of 190 patients (75.1%) were treated with H1 receptor antagonist alone, while 50 patients (19.8%) received two H1 receptor antagonists or more. In addition, nine patients (3.6%) were treated with both H1 and H2 receptor antagonists, and 21 patients (8.3%) were treated with an H1 receptor antagonist as well as a leukotriene receptor antagonist. No patients required systemic corticosteroids for ≥ 3 consecutive days or anti-IgE treatment.

Univariable analysis showed that patients who did not have inducible factors (CSU) or were not sensitized to common allergens had a better prognosis than those who had CIU or were sensitized to allergens (P = 0.021 and P = 0.010, respectively) (**Table 2**). In Kaplan-Meier survival analysis, 40.7%, 60.9%, and 78.3% of CSU recovered at 6, 12, and 24 months after symptom onset, respectively. In addition, 29.4%, 49.4%, and 67.8% of CIU patients recovered at 6, 12, and 24 months,

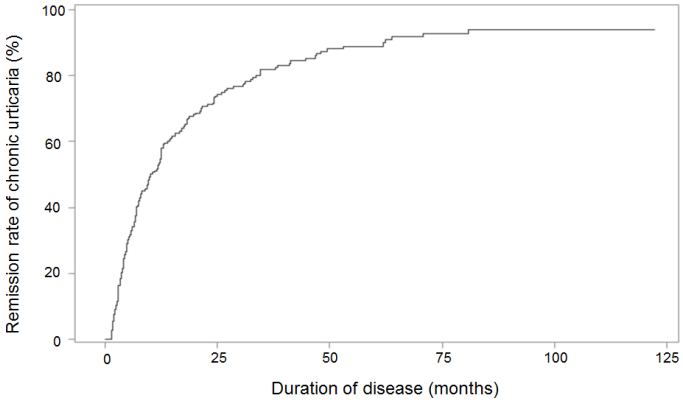

Figure 1. Kaplan-Meier survival analysis of children with chronic urticaria. Remission percentages at 6, 12, and 24 months after symptom onset were 33.4%, 53.0%, and 71.2%, respectively.

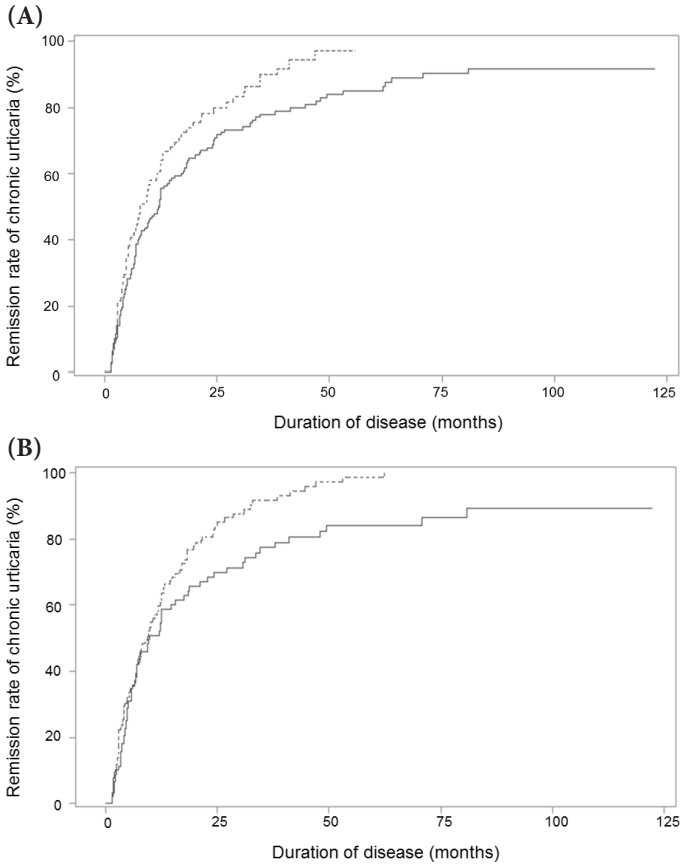

Figure 2. (A) Kaplan-Meier analysis of CIU (solid line) and CSU patients (dashed line). (B) Kaplan-Meier analysis of CU patients sensitized (solid line) and non-sensitized (dashed line) to allergens. CIU: chronic inducible urticaria, CSU: chronic spontaneous urticaria, CU: chronic urticaria.

respectively (**Figure 2**). Median disease duration was 8.0 months for children with CSU and 12.1 months for those with CIU, and 9.4 months for non-sensitized patients and 9.8 months for sensitized patients. Median recovery time was 9.6 months for boys, 10.8 months for girls, 9.6 months for younger children (< 5 years old), and 12.0 months for older children (≥ 5 years

Table 2. Predictors of remission of chronic urticaria according to univariable and multivariable Cox analyses.

Variables	Univariable analysis			Multivariable analysis		
	Hazard ratio	95% CI	*P* Value	Hazard ratio	95% CI	*P* Value
Gender						
Female	1					
Male	0.945	(0.712-1.255)	0.697			
Age						
< 5 years	1			1		
≥ 5 years	1.276	(0.962-1.692)	0.092	1.006	(0.740-1.366)	0.970
Clinical symptoms						
Wheals only	1			1		
Angioedema with wheals	1.383	(0.861-2.222)	0.180	1.393	(0.837-2.320)	0.202
Past history of allergic diseases						
No	1					
Yes	1.171	(0.879-1.563)	0.281			
Past history of autoimmune diseases						
No	1			1		
Yes	2.451	(0.907-6.623)	0.077	1.698	(0.520-5.556)	0.381
Family history of allergic diseases						
No	1					
Yes	1.068	(0.770-1.479)	0.693			
Inducible factors						
No	1			1		
Yes	1.422	(1.054-1.919)	0.021	1.330	(0.965-1.835)	0.081
Sensitization						
Negative	1			1		
Positive	1.511	(1.104-2.070)	0.010	1.486	(1.083-2.037)	0.014
Total IgE						
< 200 kU/L	1					
≥ 200 kU/L	1.093	(0.791-1.511)	0.590			
Eosinophils						
< 4%	1					
≥ 4%	1.059	(0.756-1.486)	0.737			
ESR						
< 20 mm/h	1					
≥ 20 mm/h	0.745	(0.436-1.274)	0.282			
CRP						
< 0.5 mg/dl	1					
≥ 0.5 mg/dl	0.669	(0.398-1.122)	0.128			
ANA						
Negative	1					
Positive	1.733	(0.920-3.268)	0.089			

ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; ANA, anti-nuclear antibody.

old). However, there were no significant differences in disease duration with respect to gender or age ($P = 0.697$ and $P = 0.092$, respectively) (**Table 2**). In addition, no differences were found in CU prognosis according to the presence of angioedema, past medical history, family history of allergic diseases, or laboratory findings such as total IgE level, eosinophilia, ESR, CRP level, or ANA status.

Multivariable analysis also showed that allergic sensitization was associated with the persistence of CU ($P = 0.014$), and a trend for an association between the presence of inducible factors and remission was seen ($P = 0.081$) (**Table 2**). There were no differences between patients who went into remission and those that did not with respect to gender, age, the presence of angioedema, past medical history, family history of allergic diseases, or laboratory findings.

Discussion

In our present study, we found that half of children with CU achieved remission at 10.2 months after disease onset. Overall, 33.4%, 53.0%, and 71.2% of patients achieved remission within 6, 12, and 24 months of disease onset. In addition, the symptoms of about 75% of subjects improved in response to avoiding inducible factors or taking a regular dose of H1 antihistamines, indicating a good response to conservative or

medical treatment. To our knowledge, this is the largest study to describe the natural course and prognostic factors of CU in Korean children. The percentages of patients in remission at different time points in the present study are inconsistent with those reported in previous studies.[15-20] Kang et al. reported a remission rate of 84.8% in Korean children with CU at the 1 year follow-up, which is a better prognosis than what we found in our study.[15] However, previous studies conducted in other countries reported poorer outcomes than those of our study and Kang et al.'s study. For example, a Turkish study reported that 16.5%, 38.8%, and 50.0% of children with CSU showed an improvement in symptoms within 1 year, 3 years, and 5 years after disease onset, respectively.[20] In Japanese and Thai studies, symptoms improved in 36.6% and 18.5% of patients, respectively, within 1 year.[16,18]

Disagreement among studies on the natural course of CU might be due to differences in the study population and the presence of inducible factors. In particular, a younger age is likely to be associated with a better prognosis.[15,20] In our study, the median age of patients was 5 years old, which is younger than the 8-9 years described in previous studies.[18-20] In addition, gender might affect the prognosis of CU, because sex hormones are involved in the action of mast cells, peripheral basophil cells, and dendritic cells.[4,21] In our study, the male to female ratio was 1.3: 1, which is a higher male predominance than in other studies.[18,22] According to previous reports, 50-80% of CU patients have CSU, and only 25-40% of CU patients have inducible factors, of which physical urticaria is the most common.[22-24] In our study, 31.2% of the patients did not have any cause of urticaria, despite detailed history taking and several tests. The only cause of CIU was physical urticaria, and dermographism was the most common cause of physical urticaria, consistent with another domestic study that reported that 42.3% of CU patients have dermographism.[15] In the present study, the proportion of CSU was lower than that reported in a previous study.[15] We should note that our study included dermographism as an inducible CU factor, whereas previous studies did not.

Allergic sensitization was the only factor that was associated with a worse CU prognosis in our present study, although the importance of atopic status in the pathogenesis of CU is still a matter of debate. A previous Korean study showed that children with CU had a higher prevalence of sensitization against house dust mites and polysensitization compared to those with acute urticaria.[25] Several previous studies have reported a higher prevalence of sensitization to variable allergens in patients with CU than in normal healthy subjects,[26-28] while fewer studies have investigated the association between atopy and CU prognosis. Recently, Song et al. reported that house dust mite sensitization as well as a positive autologous serum skin test (ASST) result were related to more severe disease and a longer disease duration.[26] Furthermore, higher expression of CD63 was observed in basophils from patients with CSU, suggesting that spontaneous basophil activation may contribute to the relationship between CU and allergic sensitization.[29]

The presence of inducible factors and/or autoimmune antibodies have been considered poor prognostic factors in previous studies of patients with CU, although this is still controversial.[15,22,23] We found a trend for an association between the presence of inducible factors and remission without statistical significance in the multivariable analysis. A previous study found that symptom duration was longer in autoimmune urticaria, which was defined in that study as positive ASST, than CSU.[23] In that study, 17% of patients with CU were ANA positive, and 16% and 12% of patients were positive for anti-thyroglobulin antibodies and anti-microsomal antibodies, respectively.[23] These autoantibodies could activate basophils and mast cells to induce histamine release by complement.[30] In our present study, the ANA-positive rate was 25.0%, which is higher than that reported previously for healthy children (6-14%).[31,32] However, we did not find an association between CU prognosis and autoimmune diseases, because the number of patients who received tests for autoantibodies was too small for robust statistical evaluation of this relationship. Further studies are required to identify additional prognostic factors in children with CU.

Our study had some limitations that must be considered. This study was performed in a single tertiary center, therefore selection bias might have occurred. Secondly, remission state was determined by telephone survey for some patients because of the retrospective nature of the study. There might be patients with persistent disease who were selectively missed, although the telephone survey was done with all caregivers whose children were lost to follow-up. In addition, the same laboratory tests were not performed in all patients. Therefore, some variables such as CRP and ANA were not included in the multivariable analysis. It also should be noted that the treatment in our present study followed Korean Expert Opinion Report on the Management of CU, not international guidelines.[6] Despite these limitations, we provide useful information regarding the natural course of CU in children, the response rates to medical treatment, and prognostic factors.

In conclusion, the median duration of CU in Korean children was 10.2 months, and half of the CU patients showed symptom improvement within a year, indicating that children with CU had a favorable outcome. Allergic sensitization was associated with a poor CU prognosis.

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ORIGINAL ARTICLE

Assessment of small airway function and reversibility in symptom-controlled asthma in pediatric patients

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Abstract

Background: The goals of asthma management aim to control the symptoms and minimize future risk. There is, however, an option to stop controller medication if the patient has been well-controlled for at least 6-12 months. To assess control, both clinical symptom assessment and lung function should be monitored periodically. In practical clinical practice of pediatric patients, lung function is not available at all health centers.

Objectives: to determine lung function with a focus on small airway function and the risk of reversibility among children who have been symptom-controlled.

Methods: Our participants were symptom-controlled asthmatic children according to GINA Guideline for at least 6 months with low dose inhaled corticosteroid. Written informed-consent was given by the parents and the children. They performed a self-evaluated symptom-controlled test (C-ACT) and a spirometric assessment. Abnormal lung function was defined as FEV_1 < 80%, FEV_1/FVC < 80%, and FEF_{25-75} < 65% predicted. Airway reversibility was determined by the change of FEV_1 > 12% and FEF_{25-75} > 30% post bronchodilator.

Results: Forty children (65% male) were enrolled. Age ranged between 6.7 and 15.0 years. The mean C-ACT score was 25.2 ± 1.7. Spirometry results were: mean FEV_1 84.0%, FEV_1/FVC 87.8%, and FEF_{25-75} 85.5% predicted. Normal FEV_1 was found among 72.5% of participants compared to normal FEF_{25-75} in 87.5%. Among the abnormal FEV_1 and FEF_{25-75}, all were of mild severity as 10% retained airway reversibility.

Conclusion: Children with well-controlled asthma, based on their symptom assessment, may have persistent abnormal lung function. Spirometry should be performed before considering cessation of controller medication.

Key words: small airway function, symptom-controlled, asthma, pediatric, spirometry

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Introduction

According to the terms of the Global Initiative for Asthma, the long-term goals for asthma management are to achieve good symptom control, and to minimize future risk of exacerbations, fixed airflow limitation, and side-effects of treatment. Symptom control is assessed using the frequency of daytime & night-time asthma symptoms, reliever use, and activity limitation. Poor symptom control is a risk factor for future exacerbations. Lung function, which mainly focuses on FEV_1 and the FEV_1/FVC ratio, should be assessed as an indicator of future risk.[1]

It is well-established that inflammation in asthma involves not only the large airways, but also the small airways; the complete airway tree.[2-3] Small airway inflammation is recognized as having an important role in controlling asthma. Increasing evidence suggests that small-airway dysfunction is associated with the clinical features of asthma: such as poor control of asthma and higher numbers of exacerbations.[4] In addition, significant bronchodilator reversibility in a patient taking controller treatment also suggests uncontrolled asthma.[1]

When asthma is well-controlled, complete cessation of inhaled corticosteroids (ICS) in adults is not advised as the risk of exacerbations is increased.[1,5] Nevertheless, a physician may consider stopping controller treatment only if there have

been no symptoms for 6-12 months and the patient has no risk factors.[1] By comparison, in clinical pediatric practice, routine lung function at each visit is difficult to perform. Moreover, spirometry is not available at all health care centers in our country (Thailand), so assessment of asthma symptom control is accomplished for reviewing the treatment and controller cessation. Even if children have clinical symptom control, the future risk of exacerbations and lung function (especially of the small airways) are unknown. We, thus, conducted the current study to determine lung function with a focus on small airway function as well as the risk of reversibility among children who have been assessed as symptom-controlled.

Methods

We conducted a prospective, descriptive study in children diagnosed with asthma according to the GINA guideline, and who regularly attended the Pediatric Asthma Clinic at Srinagarind Hospital, Khon Kaen University, between August 2015 and August 2016. At each visit, the patients were assessed for symptoms and those who met the criteria for symptom -controlled were invited to participate in the study. This study was approved by the Ethics Committee for Human Research of Khon Kean University, Thailand (HE581314)

Study populations

The enrolled asthmatic children were: 1) between 6 and 15 years of age and regularly attended the clinic; 2) symptom -controlled for at least 6 months [as per the GINA 2015 clinical assessment]; 3) regularly used low-dose inhaled controllers (Budesonide ≤ 200 µg/day or Fluticasone ≤ 200 µg/day plus LABA)[1]; and, 4) able to perform spirometry. Each child participant had informed, written parental consent as well as giving their own assent. Children who had had a respiratory infection in the previous 1 month and had a history of using bronchodilator before performing spirometry within 6 hours were excluded. The symptom control score for the last 1-month of symptoms was determined using the childhood asthma control test (C-ACT).[6] Parents and children who met all the eligibility criteria were asked to perform the C-ACT test before performing spirometry.

Symptom-controlled (GINA clinical assessment)[1] was defined as exhibiting none of the following: daytime symptoms more than twice/week; any night waking due to asthma; any reliever needed for symptoms more than twice/week; and, any activity limitation due to asthma in the past 4 weeks.

Childhood asthma control test (C-ACT)[6] is a seven-item assessment questionnaire, completed by the child and parent/caregiver, for assessing asthma control in children between 4 and 11 years of age in the preceding four weeks: the total score ranges between 0 and 27. The C-ACT test has been translated into Thai; however, it has not been validated in Thai children.

Pulmonary and small airway function

Lung function was measured using a CHEST multifunctional spirometer HI – 801. Typical FEV_1 and FEV_1/FVC were evaluated to determine large airway function, and FEF_{25-75}—the latter reflecting small airway function. Repeated spirometry was performed 15 minutes after giving inhaled Ventolin Evohaler® plus spacer to determine reversibility of the airway. Abnormal lung function was defined as FEV_1 < 80% predicted, FEV_1/FVC < 80%, and FEF_{25-75} < 65%. Post bronchodilator, the change of FEV_1 > 12% and FEF_{25-75} > 30%, was positive for reversibility.[7-8] Spirometry was performed by the same trained nurse throughout the study. We documented the demographic characteristics, BMI, family history of atopy, co-morbidity, time of asthma diagnosis, and treatment.

Study analysis

Statistical analyses were performed using SPSS version 19. Continuous data were presented as means ± SD, medians, and ranges (minimum to maximum). Categorical data were presented as numbers and percentages of each group.

Results

There were 40 patients who met the eligibility criteria. There were 26 (65%) males and overall the age ranged between 6.7 and 15.0 years (mean, 10.4 ± 2.0). BMI ranged between 12.4 and 27.2 (mean, 18.3 ± 3.8 kg/m^2). No child was diagnosed as obese (i.e., BMI ≥ 95th percentile). Half of the patients had a co-morbidity with the allergic rhinitis. Sixteen (40%) had a family history of smoking by the father and/or grandfather. Other baseline characteristics included mean duration of diagnosed asthma (6.0 ± 3.2 years) and mean duration of taking ICS (5.6 ± 3.3 years). Half of the patients had had a history of admission due to asthma exacerbation prior to being symptom-controlled (average, 2 times). (**Table 1**) No child had severe

Table 1. Demographic characteristics of the studied population

Characteristic	Total N = 40
Sex, male (%)	26 (65.0)
Age, (years)	
mean ± SD	10.4 ± 2.0
median (min - max)	10.4 (6.7 - 15.0)
BMI, (kg/m^2)	
mean ± SD	18.3 ± 3.8
median (min - max)	18.1 (12.4 - 27.7)
Birth weight, (g)	
mean ± SD	3,160.6 ± 337.7
median (min - max)	3,175.0 (2,100 - 3,750)
Breastfeeding, (months)	
mean ± SD	3.1 ± 2.0
median (min - max)	3.0 (1 - 8)
Family history of smoking, n (%)	16 (40.0)
History of allergic rhinitis, n (%)	20 (50.0)
History of admission prior to symptom-controlled, n (%)	20 (50.0)
mean number of admissions	2.1 ± 1.8
median (min - max)	1.0 (1 - 6)
History of severe exacerbation or admission in the past year	0

exacerbation or admission during the past year. The mean C-ACT score was 25.2±1.7. The range of C-ACT for most children was 22 to 27. Only one child had a score as low as 19. Additional diagnoses and treatment are shown in **Table 2**.

Lung function outcomes were: mean of predicted FEV_1 84.0 ± 8.1%, FEV_1/FVC 87.8 ± 6.1%, and FEF_{25-75} predicted 85.5 ± 20.0%. Normal large airway function (FEV_1 ≥ 80%) was found in only 72.5% of participants while normal small airway function (FEF_{25-75} ≥ 65%) was found in 87.5%. Among the abnormal FEV_1 and FEF_{25-75}, all were of mild severity (FEV_1 > 70% predicted) (**Table 4**). All children who had an abnormal FEV_1 and FEF_{25-75} had a C-ACT score between 22 and 27.

The post-bronchodilator results showed airway reversibility as follows: FEV_1 returned to a normal predicted level in 34 patients (85%) while most of the abnormal FEF_{25-75} returned to a normal predicted level in 39 patients (97.5%). Among the children with normal lung function, 4 (10%) showed airway reversibility: 2 had both large airway (FEV_1 > 12%) and small airway reversibility (FEF_{25-75} > 30%) and another 2 had only small airway reversibility (FEF_{25-75} > 30%). (**Table 5**) The clinical characteristic of these 4 children are presented in **Table 3**. As for the risk of exacerbation, two had allergic rhinitis as co-morbidities with a history of smoking in the family. Three of them received the maximum dose of ICS at a medium level

Table 2. Treatment and symptom control of asthma prior to enrollment

Disease characteristic	
Onset of suspected asthma symptoms (years)	
mean ± SD	3.5 ± 3.2
median (min - max)	2.0 (0.1 - 12.0)
Age at asthma diagnosis (years)	
mean ± SD	4.3 ± 3.2
median (min - max)	4.0 (0.8 - 12.0)
Duration of asthma (years)	
mean ± SD	6.0 ± 3.2
median (min - max)	5.8 (0.8 - 11.2)
Duration of taking ICS (years)	
mean ± SD	5.6 ± 3.3
median (min - max)	5.1 (0.8 - 11.2)
Duration of controlled symptoms (years)	
mean ± SD	1.7 ± 2.3
median (min - max)	1.0 (0.5 - 10.0)
Maximum daily dose of controller prior to symptom-controlled, n (%)	
Budesonide > 400 µg/day or Fluticasone > 250 µg/day + LABA	27 (67.5)
Budesonide ≤ 400 µg/day or Fluticasone ≤ 250 µg/day + LABA	13 (32.5)
C-ACT score	
mean ± SD	25.2 ± 1.7
median (min - max)	25.0 (19 - 27)

Table 4. Spirometric results

Spirometric results	Pre BD	Post BD
FEV_1		
Mean ± SD	84.0 ± 8.1	86.5 ± 8.6
Median (min - max)	84.3 (69.1 - 108.1)	85.9 (61.2 - 105.9)
≥ 80% (normal), n (%)	29 (72.5)	34 (85)
FEV_1/FVC		
Mean ± SD	87.8 ± 6.1	90.6 ± 5.4
Median (min - max)	87.7 (71.4 - 100)	91.2 (76.4 - 100)
≥ 80% (normal), n (%)	37 (92.5)	38 (95)
FEF_{25-75}		
Mean ± SD	85.5 ± 20.0	99.7 ± 21.9
Median (min - max)	83.2 (46.8 - 152.2)	96.4 (58.1 - 162.9)
≥ 65% (normal), n (%)	35 (87.5)	39 (97.5)

Table 5. Post bronchodilator spirometric results

Lung function	FEV_1	Reversible N (%)	FEF_{25-75}	Reversible N (%)
Normal N (%)	29 (72.5)	2 (5)	35 (87.5)	4 (10)
Abnormal N (%)	11 (27.5)	0	5 (12.5)	0

Table 3. Clinical characteristics of the subjects who had airway reversibility

Characteristic	Subject 1	Subject 2	Subject 3	Subject 4
FEV_1 reversibility (% change > 12%)	Yes	No	Yes	No
FEF_{25-75} reversibility (% change > 30%)	Yes	Yes	Yes	Yes
Age of asthma onset (year)	0.8	5	1.4	7
BMI (kg/m^2)	16.6	21.1	13.3	17.6
Co-morbidity	No	Allergic rhinitis	No	Allergic rhinitis
Family history of asthma in 1st degree relative	No	No	No	No
Passive smoker	Yes	No	Yes	No
History of severe exacerbation in the past year	No	No	No	No
Type of controller to achieve asthma control	Fluticasone + LABA	Fluticasone + LABA	Fluticasone + LABA	Budesonide
Maximum dose of controller needed to achieve asthma control (µg/day)	500	250	500	400
C-ACT score	26	22	26	25

but none experienced a severe exacerbation or admission in the past year nor were they activity-limited. All of them achieved asthma-control and the ICS could be stepped down to a low dose for more than 6 months, as required before enrollment.

In the abnormal lung function group, none showed any airway reversibility. One child had both mild fixed abnormal FEV_1 and FEF_{25-75}. (Pre & Post BD FEV_1 = 71.4 & 75%, FEF_{25-75} = 46.8 & 58% predicted, respectively). The child had a BMI of 27.3 kg/m^2 and had been diagnosed with asthma at 5. He had been on ICS for 5 years without any severe exacerbation, but was a passive smoker from his father since birth and his C-ACT score was 26.

Discussion

This study was conducted to test the hypothesis that all asthmatic children who have achieved symptom controlled with low-dose ICS for at least 6 months have normal lung function of the entire airway but in particular of the small airway. As for asthma management, all of the patients not only have to achieve good symptom control, the future risk of exacerbations should be minimal. One of the important future risks is low FEV_1. In addition, significant bronchodilator reversibility in a patient taking controller treatment suggests uncontrolled asthma.[1] The current study showed that some children who were supposed to be well-controlled according to their symptom assessment, continued to have both abnormal large and small airway function.

Several studies have been conducted to determine the correlation of symptom assessment and lung function during the treatment follow-up period. Munoz et al. demonstrated that eosinophilic or neutrophilic inflammation persisted in most well-controlled asthma patients despite their condition being controlled.[9] Recent studies, moreover, showed that small airway dysfunction is not only a feature of severe asthma but can also present in patients with mild asthma who have a low level of symptoms and normal FEV_1 values.[10-13] Huang et al. demonstrated that the majority of children with well-controlled asthma continued to have airway hyper-responsiveness and low small airway function as represented by FEF_{25-75}.[14]

Many physiological and imaging techniques have been used to evaluate small airway function, including impulse oscillometry, exhaled nitric oxide, inert gas washout, high resolution computed tomography, and spirometry.[15] Spirometry is often readily available and used most frequently in routine clinical practice. Forced expiratory volume in one second (FEV_1) and FEV_1/forced vital capacity (FVC) mainly represent the larger airways, whereas forced expiratory flow between 25% and 75% of forced vital capacity (FEF_{25-75}) reflects small airway function.[15] Rao et al showed that asthmatic children who had a low FEF_{25-75} had nearly 3 times the odds (OR 2.8) of systemic corticosteroid use and 6 times the odds of asthma exacerbations (OR 6.3) compared with those who had normal spirometry. In addition, they also concluded that using the percent change in FEF_{25-75} may be helpful in identifying bronchodilator responsiveness in asthmatic children with a normal FEV_1.[8] Due to our limited resources, we used spirometric results (FEF_{25-75} predicted) to represent small airway function and pre-post bronchodilator values to determine airway hyper-reactivity. We found that FEF_{25-75} did not add more useful information for the detection of abnormal lung function. Nevertheless, among children who had a normal FEV_1, four (10%) continued to have airway reversibility especially of the small airway. Prior to being considered symptom-controlled, these four children were treated with ICS at a medium dose level. Regarding clinical assessment of future risk of exacerbation, they did not have any striking history, such as a history of severe exacerbation during the preceding year, no activity limitations or C-ACT score between 22-26. Two children had allergic rhinitis as a co-morbidity and environmental smoking exposure. Notwithstanding these clinical characteristics, they were similar to children with normal lung function. The finding of significant bronchodilator reversibility (i.e., an increase in FEV_1 > 12%, FEF_{25-75} > 30%)[16] in a patient taking controller treatment suggests persistent uncontrolled asthma, which should be regarded as evidence for further continuing treatment.[1] Early controller cessation in these patients might lead to severe exacerbation and fixed airway obstruction in the future. This study confirmed the usefulness of assessment of small airway reversibility to determine asthma control status in children.[8,16]

The recent international practice guideline for asthma recommends that lung function—especially spirometry—is a useful indicator of exacerbation, so it should be monitored together with clinical assessment at the start of treatment, after 3-6 months of controller treatment, and periodically going forward.[17-18] Monitoring asthma management in children according to these guidelines is challenging. In general, symptom control assessment as provided by GINA is used routinely for control-based asthma management at most healthcare centers in our country. Nevertheless, several asthma control scores for children—i.e., Chilhood Asthma Control Test (C-ACT)—have been developed to help in clinical assessment.[6] Medication adjustment as well as cessation of controller mainly depended on subjective symptom control assessments. The current study used the GINA assessment at the level of well-controlled as indicating symptom control.[1] We also compared the GINA assessment with the C-ACT score and found that most children who were well-controlled had a C-ACT score in the range of 22 to 27. Only one child whose lung function (both FEV_1 and FEF_{25-75}) was normal had a C-ACT score of 19. The results of our study on symptom-control assessment are similar to the study by Koolen et al. who showed that a C-ACT score correlated well with the GINA criteria and that children who were well-controlled had a C-ACT score in the range of 23 to 27.[19] By comparison, Ito et al. showed that a C-ACT cut-off score of 23 was useful for identifying children with well-controlled asthma (sensitivity 78%; specificity 54%).[20] This C-ACT score has been translated into Thai but is not widely used possibly because it has not yet been validated.

The European Task Force recommends performing spirometry annually as a minimum, when monitoring asthma in children.[18] The results of our study confirmed the usefulness of objective monitoring in asthma patients especially spirometry; however, in our country, spirometry is not available at all levels of health care. The current study demonstrated that even if the clinical assessment (either GINA or C-ACT score) of asthmatic children indicates symptom controlled', their lung function might not be normal and they may even have airway reversibility. We, therefore, suggest that before considering

cessation of controller medication in children over 6 with well-controlled asthma, a lung function test be performed to demonstrate the actual status, if available.

The limitations of this study were that (a) symptom control assessments were done by several pediatricians caring for the patients, so there will be some variation in assessments; (b) no inter-rater variation was performed before enrollment; and (c) we had a small sample size. In addition, it was a cross-sectional study that might not be able to determine the exact future clinical outcomes. Further study with an adequate sample size and long term cohort should be performed to add more evidence to support the clinical practice guideline.

Conclusion

The current study demonstrated that asthmatic children, who are considered symptom-controlled based on an assessment of their treatment and symptoms, may still have abnormal lung function. Spirometry should be performed before considering cessation of controller medication.

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ORIGINAL ARTICLE

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Beta-Expansin of Bermuda, Johnson, and Para grass pollens, is a major cross-reactive allergen for allergic rhinitis patients in subtropical climate

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Abstract

Background: Subtropical grass pollens of Bermuda (BGP), Johnson (JGP), and Para or buffalo grass (PGP), are common causes of pollen allergies in warm climate area. Allergic rhinitis (AR) patients had positive skin prick test (SPT) to extract of these 3 grass pollens. However, no allergenic proteins of 3 grass pollens have never been studied.

Objective: To identify major allergens of BGP, JGP, and PGP in Thai grass pollen-allergic patients and to examine their sIgE cross-reactivity.

Methods: Serum of nine AR patients with positive SPT to at least 2 of 3 studied pollens were collected. Based on availability, only ImmunoCAP of BGP and JGP were available to determine a level of sIgE. Profiles of sIgE bound proteins from BGP, JGP, and PGP, were obtained by immunoblot. Major IgE bound protein was identified by liquid chromatography-tandem mass spectrophotometry (LC-MS/MS). Cross-reactivity of purified major allergen of the 3 grass pollens was determined by inhibition of sIgE in both ELISA and immunoblot.

Results: AR patients who have positive SPT to extract of BGP, JGP, and PGP, were 9, 8, and 6, respectively. Positive sIgE (> 0.35 kUA/L) to BGP and JGP were found in 9 and 8 patients, respectively. Eight profiles of IgE bound proteins of the 3 grass pollens showed 29-30 kDa pollen protein as major allergen and was identified as beta-expansin (ExpB). Moreover, purified ExpB of the 3 grass pollens cross-inhibited serum sIgE.

Conclusion: Approximately 30 kDa ExpB of BGP, JGP, and PGP, are major cross-reactive allergen for AR Thai patients.

Key words: Subtropical grass pollen allergens, pollen allergy, Allergic rhinitis, beta-expansin, allergic airway diseases, major airborne pollens

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Introduction

Allergens from grass pollens (GP) are one of major causes of allergic airway diseases, especially allergic rhinitis (AR).[1,2] Subtropical grasses in Chloridoideae subfamily (Bermuda grass, B, or *Cynodon dactylon*) and in Panicoideae subfamily (Johnson grass, J, or *Sorghum halepense*, and Para or Buffalo grass, P, or *Brachiaria mutica*, also known as *Urochloa mutica*), are commonly distributed in warm regions such as parts of Africa, Asia, Australia, United States of America (California and Florida), Central and South America.[2] BGP, JGP, and PGP are reported as a source of allergenic proteins triggering allergic inflammation in upper airway. To date, 7 allergens of BGP and 2 allergens of JGP have been reported.[2] However, to date, allergens of PGP have not yet been identified. In Thailand, pollens of grasses are one of major airborne pollens found in a high count throughout a year and are highest count in May

and September for grass pollen ≤ 40 μm and in September for grass pollen > 40 μm.[3] It has been reported that, based on skin prick test (SPT), 52.3% and 49.4% of 736 Thai patients attending the ENT Allergy Clinic at Siriraj Hospital, had reaction to protein extract of BGP and PGP, respectively.[1] Moreover, It was recently reported that, based on SPT results, 45.6% of 68 Thai volunteers with respiratory allergy had reaction to JGP extract.[4] These findings indicated that allergens of BGP, JGP, and PGP, are one of major causes for respiratory allergy in sensitized Thai patients. Despite these results, however, pollen allergens from these 3 pollens sensitized by Thai patients have not yet been identified. Therefore, this study aimed to identify major allergenic proteins of BGP, JGP, and PGP, sensitized by Thai pollen-allergic patients as well as examine their cross-reactivity.

Methods

Patients

The study was approved by the Institutional Review Board, Siriraj Hospital (SiEc 100/2012). Forty patients with history of AR were recruited. All patients signed an informed consent before SPT. Thirty-one of them were excluded due to mono -sensitization to grass allergen. Nine patients, aged 9-47 years old (mean 26.3 ± 4 years), were included in this study based on positive SPT (mean wheal diameter, MWD ≥ 3 mm) to protein extract from at least 2 of 3 BGP, JGP, and PGP. The SPT extracts were prepared as described previously.[4] Determination of level of serum sIgE against extract of BGP and JGP was carried out by ImmunoCAP (Phadia, Uppsala, Sweden). However, there is no available test for PGP.

Pollen collection

Grass flowers were collected from various sites in Thailand. Flowers were dissected under a stereo-microscope and identified to species using identification key. Flowers were left for natural shedding for 1 day. Pollen grains were incubated for 24 h in a chamber containing silica gel before purified by passing through 120, 150, and 230 mesh-sieve. Only the pollen with 95% purity observed under the microscope were stored at -80°C until used.

Pollen crude extract preparation

Pollens were ground in liquid nitrogen and extracted in phosphate buffer saline (PBS) buffer containing 1 mM Phenylmethylsulfonyl fluoride (PMSF) with continuous stirring at 4°C for 1 h. The extract was centrifuged at 13,000 × g for 15 min and supernatant was collected before filtered through 0.45 micron filter. Protein concentration was determined using Bradford's assay. The efficacy and safety of this extract has been previously reported.[4]

Electro-elution of 29-30 kDa IgE bound pollen protein

Electro-elution was carried out using electro-eluter (Bio-Rad, USA). The preparative gels were carried in parallel with gels for immunoblot to ensure that correct protein bands were excised. The protocol was followed the instruction from the manufacturer. Twenty μg of pollen protein extract was loaded per well and resolved in 12% SDS-PAGE gel. This preparative gel was prepared with the same percentage and running condition as the gels prepared for immunoblot. Coomassie G-250 stained protein band matched IgE bound protein band of immunoblot was excised and placed in glass tube connected with pre-soaked membrane cap (3.5 kDa MWCO) where eluted protein was retained. The assembled glass tube was filled with elution buffer (25 mM Tris-HCl pH 8.2, 192 mM glycine and 0.1% (w/v) SDS) and placed into a tube holder. The holder was placed in buffer tank of Mini Transblot Electrophoresis Transfer Cell (Bio-Rad, USA) and protein was eluted from gel at a constant current of 8 mA/glass tube for 3-4 h. Eluted protein retained in the membrane cap was transferred and stored at -20°C. Analysis by SDS-PAGE gel and immunoblot were done to confirm its IgE binding activity before used in all IgE inhibition tests.

Identification of Major IgE bound protein by Liquid Chromatography-Tandem Mass Spectrophotometry (LC-MS/MS)

The following protocol was done per gel piece of one protein band. An excised gel piece was submerged in buffer A [50% Acetonitrile (ACN), 50 mM NH_4HCO_3] until colorless. The buffer A was replaced with 10 mM dithiothreitol (DTT) and incubated for 15 min at 60°C before was added with buffer B (55 mM iodoacetamide, 50 mM NH_4HCO_3) and incubated for 30 min at room temperatue in the dark. The 100% ACN was replaced to dry gel before adding trypsin solution [0.1mg/mL trypsin (Sigma-Aldrich, USA), 50 mM Ammonium bicarbonate] and incubated at 37°C overnight. The reaction was mixed with ACN at 1:1 (v/v) ratio and incubated for 20 min. All solution was transferred to a new tube and dried in centrifugal concentrator at 45°C. Resolved pellet in 0.1% formic acid was injected into an Ultimate 3000 nano-LC system (Dionex; Surrey, U.K.) coupled with Micro-ToF Q II mass spectrometer (Bruker; Bremen, Germany). The mass spectra data were acquired using Hystar software (Bruker Daltonics, Germany) and were converted by Compass Data-Analysis software (Bruker Daltonics, Germany). The converted files were analyzed with Mascot server (version 2.3.0, Matrix Science, USA) to search matched sequence in NCBI database with 95% confidence.

Direct IgE binding and Inhibition of IgE ELISA

Pollen extracts were coated at 500 ng/well of a 96-well microtiter plate and incubated at 4°C overnight. The coated plate was washed and incubated with 1/4-1/20 diluted serum of SPT positive patients and control for 2 h and washed before incubated with HRP-labeled mouse IgG anti-human IgE for 1 h. HRP substrate TMB (3,3′5,5-tetramethylbenzidine) was added and blue color reaction product was read at a wavelength of 650 nm.

For inhibition ELISA, 500 ng of eluted 30 kDa beta -expansin (ExpB) of BGP extract was coated per well. Pre -determined diluted sera of patients and control were incubated with 0.5, 1, 10, and 20 μg/ml of eluted ExpB (29-30 kDa protein) of BGP (CD29), JGP (SH29), and PGP (BM30), for overnight at 4°C before was centrifuged at 17,210 × g for 10 min at room temperature (RT). The supernatant was added to coated wells and followed the mentioned direct ELISA protocol.

Direct IgE Immunoblot and IgE inhibition immunoblot

Sera of patients and control were diluted at 1/10-1/50 in PBS containing 3% skimmed milk (buffer A) and used in the following tests.

For direct IgE immunoblot, 2 µg of pollen protein extract were resolved per well of 12% SDS-PAGE gel at constant current. Separated proteins in the gel were electro-transferred onto nitrocellulose membrane before incubated in buffer A for 1 h at RT. The membrane was washed with PBS containing 0.2% v/v tween-20 (buffer B) before incubated overnight at 4°C with diluted sera of patients and control in buffer A. The membrane was washed with buffer B and was incubated with 1:10,000 diluted horse-radish peroxidase (HRP) conjugated mouse IgG anti-human IgE antibody (KPL, MD, USA) in buffer A for 1 h. After washing, the membrane was incubated with HRP substrate (Millipore, MA, USA) and emitted signal was captured by x-ray film.

For IgE inhibition immunoblot, 1/50 diluted sera of patients and control were incubated with 20 µg of eluted ExpB for overnight at 4°C before was centrifuged at 17,210 × g for 10 min at RT. The supernatant was incubated with nitrocellulose membrane on which had transferred CD29, SH29, and BM30, for 2 h at RT and the remained steps were the same as those of direct immunoblot.

Results

Nine patients with AR were included in this study based on positive SPT to at least 2 of 3 extracts (**Table 1**). The mean age of patients was 26.3 ± 12.3 years. The severity of allergic rhinitis was not different between patients who had low or high specific IgE. Thus, all included patients were classified as mild persistent allergic rhinitis. All patients had positive SPT to BGP extract while 8 of 9 had positive SPT to JGP extract (**Table 1**). However, only 6 of 9 patients had positive to PGP pollen extract. The MWD of SPT to extract of BGP, JGP, and PGP, were 5.8 mm, 3.8 mm, and 3 mm, respectively. The results of ImmunoCAP showed all patients had sIgE > 0.35 kUA/L to BGP extract while 8 patients had sIgE > 0.35 kUA/L to JPG extract (**Table 1**).

To further determine profiles of IgE bound pollen proteins extracted from 3 grasses of all included patients, the results showed that 8 profiles had IgE bound protein mobilized between 25 and 37 kDa markers (**Figure 1**). One profile (Pt33) could not be determined due to a lack of serum. Two profiles

Table 1. Demographic of recruited Allergic Rhinitis individuals of this study

Pt. ID	sex	Age	SPT (mean wheal diameter, mm)			sIgE (kUA/L)	
			Bermuda	Johnson	Para	Bermuda	Johnson
9	F	46	9	5	3	30.3	34.7
17	M	30	4	4	2	4.16	5.19
29	M	21	5	3	2	4.02	3.05
33	M	10	8.5	4.5	3.5	>100	85.3
36	M	18	4	5	4.5	9.1	13.6
39	M	25	4	3.5	2	1.23	0.51
40	F	41	7.5	3.5	4.5	0.85	0.55
43	F	33	6.5	4	2.5	0.73	0.04
47	M	13	3.5	2	3	11.2	11.4

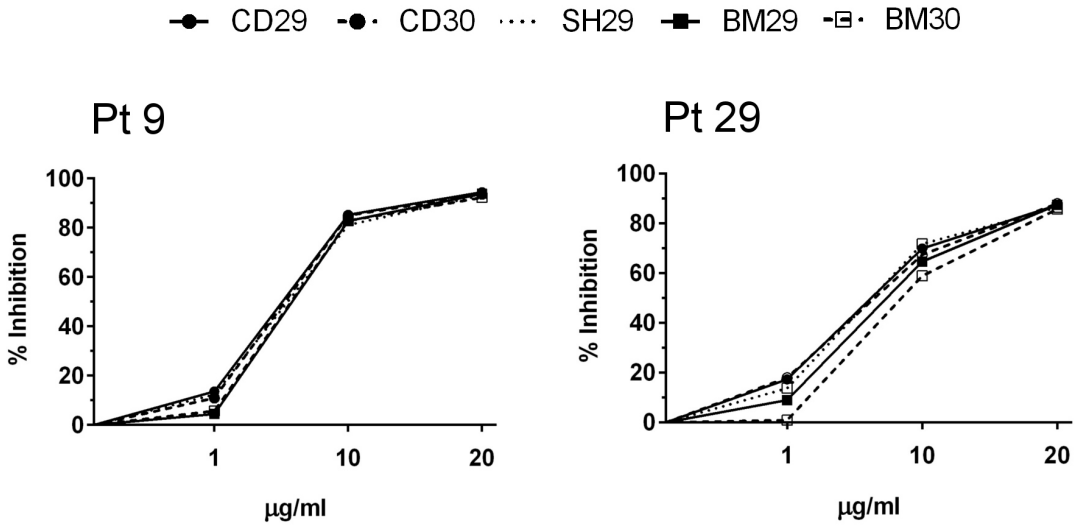

Figure 1. Profiles of IgE bound pollen proteins extracted from BGP, JGP, and PGP.
Note: total protein extract of grass pollens: 1 = BGP, 2 = JGP, and 3 = PGP.

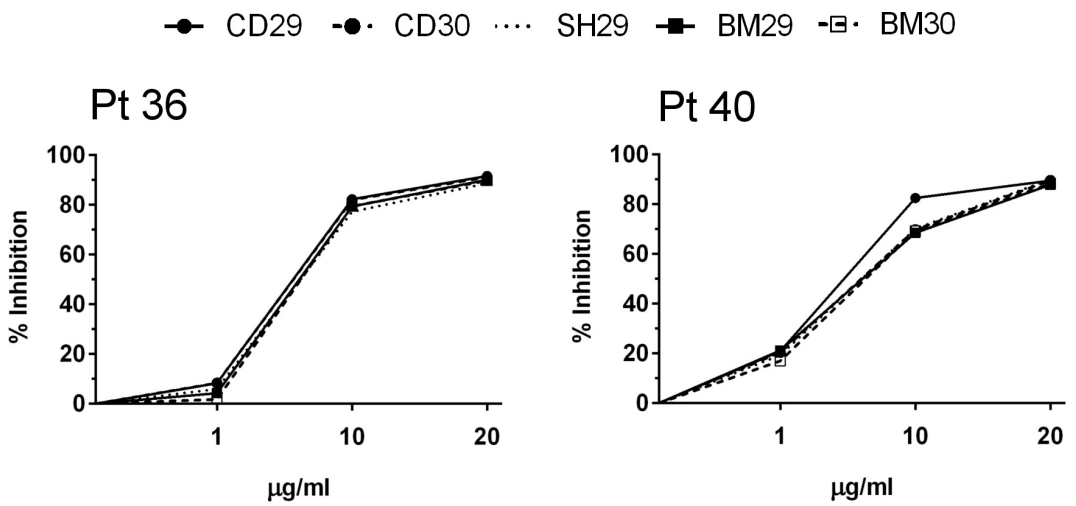

Figure 1. (Continued) Profiles of IgE bound pollen proteins extracted from BGP, JGP, and PGP.
Note: total protein extract of grass pollens: 1 = BGP, 2 = JGP, and 3 = PGP.

(Pt29 & Pt39) show IgE bound multiple proteins of 3 grass mobilized 37-50 kDa markers, while other five profiles show IgE bound multiple proteins of 1 grass mobilized 37-50 kDa markers (**Figure 1**). Moreover, three profiles (Pt17, Pt39 & Pt47) showed IgE bound multiple proteins of 3 grass mobilized 10-20 kDa markers (**Figure 1**). Based on these IgE bound protein profiles, protein mobilized between 25-37 kDa appears to be a major allergen among the 3 grass and was subjected for LC-MS to identify its identity.

The results of LC-MS/MS showed that 2 excised proteins (CD29, 30) of BGP extract matched 5-7 peptides of ExpB or Cyn d 1 (**Table 2**). Excised protein of JPG (SH29) and PGP (BM29, 30) extract matched 2-5 peptides of expansin B1 of corn (Zea mays) (**Table 2**). Moreover, the sequence of BM30 also matched 4 peptides of expansin B9 of japanese rice (*Oryza sativa subsp. japonica*) (**Table 2**). These results suggested that a protein of ExpB family is the major allergen of BGP, JGP, and PGP, sensitized by Thai AR patients. To confirm

Table 2. LC-MS identified IgE bound protein of 3 grass pollens

ID	protein	mass	Protein score	peptide sequence
CD29	Cyn d 1	26872	153	SSWGAIWR ATFYGSNPR AGELTLQFR KPLKGPFSIR KAGELTLQFR CKEPVECSGEPVLVK
CD30	Cyn d 1	26872	140	SSWGAIWR ATFYGSNPR AGELTLQFR DSDEFIPMK KPLKGPFSIR
SH29	Expansin-B1 Zea mays	29066	137	GCGSCYEVR AFGSLAKPGLNDK AFGSLAKPGLNDKIR NVNLPPYSGMTACGNVPIFK NVNLPPYSGMTACGNVPIFKDGK
BM29	Expansin-B1 Zea mays	29066	65	LSWGAIWR ALKGPFSIR AFGSLAKPGLNDK AFGSLAKPGLNDKIR
BM30	Expansin-B1 Zea mays	29066	42	LSWGAIWR AFGSLAKPGLNDKIR
	Expansin-B9 Oryza sativa subsp. japonica	28966	34	LSWGAIWR AGIIDMQFR KAGIIDMQFR AGIIDMQFRR

Note: Matching of amino acid sequence using Mascot server (version 2.3.0, Matrix Science, USA) with 95% confidence.

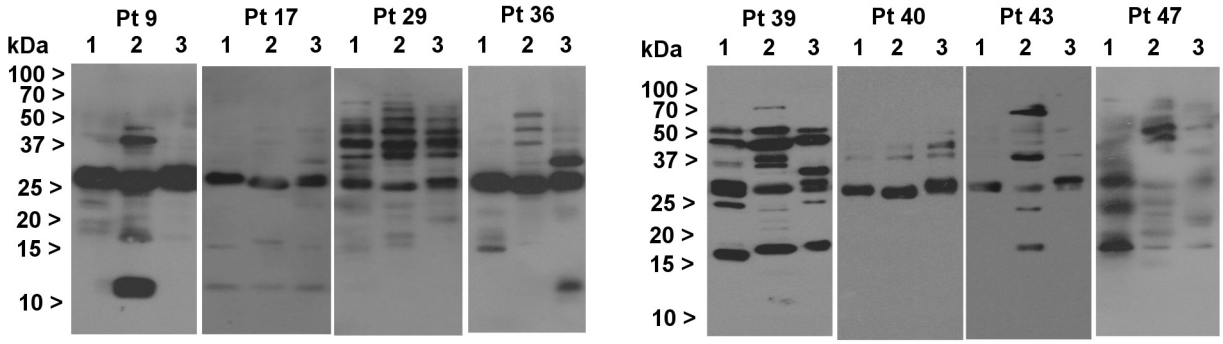

Figure 2. Cross-inhibition of specific IgE binding to coated CD29 with CD30, SH29, BM29, and BM30.
Note: serum of 4 individuals: Pt9, Pt29, Pt36, and Pt40; specific IgE binding was inhibited at an average IC_{50} of 6 µg/ml.

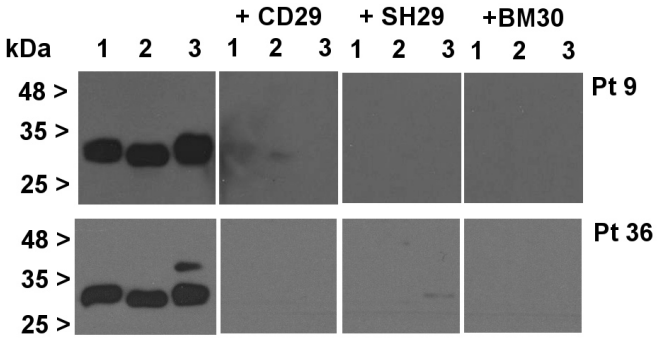

Figure 3. Inhibition of specific IgE to ExpB of 3 grass pollens with eluted ExpB.
Note: serum specific IgE of Pt9 and Pt36; 1 = eluted CD30; 2 = eluted SH29; 3 = eluted BM30; +CD29, +SH29, and +BM30 = incubated serum with eluted CD29, SH29, and BM30, respectively.

IgE cross-reactivity of ExpB of the 3 grass pollens, inhibition of sIgE binding to CD29 by CD30, SH29, BM29, and BM30, were determined. The results of inhibition ELISA showed all 5 proteins were able to cross-inhibit sIgE binding to coated CD29 at an average IC_{50} of 6 µg/ml (**Figure 2**). Moreover, the results of inhibition immunoblot also showed complete inhibition of IgE binding to CD29, SH29, and BM30 by the same 3 proteins (**Figure 3**).

Discussion

This is the first study to report that ExpB of BGP, JGP, and PGP, is major IgE cross-reactive allergen for grass pollen allergic Thai AR patients. It has been reported that ExpB or Cyn d 1 of BGP appears to be major grass pollen allergen in many subtropical areas.[5] The Cyn d 1 cross-inhibited sIgE binding to ExpB of subtropical Bahia grass (Pas n 1) with strong affinity while to that of temperate Ryegrass (Lol p 1) with moderate affinity.[5,6] The difference in IgE affinity to group 1 allergen of subtropical grass and temperate grass may be caused by different subfamily. In subtropical grasses, Bermuda is in Chloridoideae subfamily while both Johnson and Para are in Panicoideae subfamily. Both subfamilies are from the same lineage (PACMAD clade) in phylogenic tree and ExpB of the 2 subfamilies shared 65% identity of amino acid sequence. Based on the LC-MS results, identical amino acid sequences between ExpB of BGP and PGP are WGAIWR and LKGPFSIR, located in the C-terminal domain of Cyn d 1.[8,9] Moreover, the peptide, WGAIWR (aa194-199) was showed to be one of major IgG4 binding sites on Cyn d 1.[9] Despite a moderate sequence identity, ExpB of these 3 grass pollens could cross-inhibit sIgE to ExpB with strong affinity (IC_{50} of 6 µg/ml). It has been reported that IgE cross-reactivity between group 1 of subtropical grass and that of temperate grass could also trigger allergic reaction in patients from either subtropical or temperate regions.[10,11] Based on these results, thus, ExpB of one grass species, such as Cyn d 1, could be a good allergen used in diagnosis and immunotherapy of AR patients who sensitize to subtropical grass pollens from grass of PACMAD clade. Moreover, several agricultural crops, such as maize, sorghum, sugar cane, and pearl millet, are belonged to the same Panicoideae subfamily as Johnson and Para grass. Thus, investigation of IgE cross-reactivity of ExpB in pollens from mentioned agricultural crops would be essential because they are economically important crops for countries in warm regions and many field workers may likely suffer pollen-caused AR.[2]

Results of SPT and ImmunoCAP appear to correlate well as 7 of 9 patients had a value of sIgE against BGP and JGP grass pollen ≥ 0.35 kUA/L while had ≥ 3 mm MWD in SPT. However, the other 2 patients did not have all positive results from both testes, for instance, Pt8 had SPT positive to both BGP and JGP but sIgE to only BGP or, for Pt9, SPT positive to BGP but sIgE to both BGP and JGP (**Table 1**). Although values of sIgE and SPT of 7 patients indicated they are allergic to proteins of BGP and JGP, however, values of these 2 tests may not predict results of one another. For instance, Pt4 had SPT value of 8.5 mm to BGP and 4.5 mm to JGP,

while sIgE values were > 100 and 85.3 kUA/L to BGP and JGP, respectively. In contrast, Pt7 had SPT value of 7.5 mm to BGP and 3.5 mm to JGP, while sIgE values were 0.85 and 0.55 kUA/L to BGP and JGP, respectively. Based on these results, it appears that SPT could be sufficient test to diagnose pollen allergy as also reported by others.[1,4-7,10,11] Pollen extract in this report was prepared from 95% purified pollens from identified grass species.[4] Therefore, results from SPT and IgE reactivity assays, such as ELISA, would be a result from allergens interacting sIgE.[1,4-7,10,11]

In conclusion, ExpB of Bermuda, Johnson, and Para grass pollens is the major cross-reactive allergen for Thai patients with AR and could be one of essential components for diagnosis of pollen allergy.

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ORIGINAL ARTICLE

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Lactobacillus plantarum induces genomic DNA-dependent and TLR9-mediated elafin secretion from Caco-2 cells

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Abstract

Background: Lactobacilli show anti-inflammatory effects in the human intestine, and their genomic DNA was identified as one of the anti-inflammatory components. Increased levels of the natural protease inhibitor elafin in the intestine plays an important role in protection against intestinal inflammation. However, there have been no previous reports regarding whether lactobacilli increase elafin levels.

Objective: This study was performed to investigate whether *Lactobacillus plantarum* induces elafin secretion from the human epithelial colorectal adenocarcinoma cell line, Caco-2. Moreover, we examined the roles of bacterial genomic DNA and toll-like receptor 9 (TLR9), a specific receptor of bacterial DNA, in this effect.

Methods: Elafin secretion from Caco-2 cells by live and heat-killed *L. plantarum* was measured. The analysis was also performed using DNase-treated *L. plantarum* and genomic DNA extracted from *L. plantarum*. We examined the role of TLR9 in elafin secretion by *L. plantarum* and its genomic DNA by suppressing TLR9 expression using RNAi in Caco-2 cells.

Results: Heat-killed *L. plantarum* time- and dose-dependently increased elafin secretion, whereas live *L. plantarum* had no such effect. The elafin secretion by heat-killed *L. plantarum* was partially abolished by DNase treatment of the bacterium. In addition, *L. plantarum* genomic DNA also increased elafin secretion. Furthermore, suppression of TLR9 expression partially or completely abolished elafin secretion by heat-killed *L. plantarum* and its genomic DNA.

Conclusion: Our results indicated that heat-killed *L. plantarum* induced genomic DNA-dependent and TLR9-mediated elafin secretion. The anti-inflammatory effects of lactobacilli may be mediated by increases in the levels of elafin in the intestine.

Key words: elafin, *Lactobacillus plantarum*, genomic DNA, toll-like receptor 9, anti-inflammatory effect

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Introduction

Lactobacilli, which are normal components of the human gut microflora, have been used as both foods and medicines because of their beneficial effects on the health of the host. For example, treatment with lactobacilli has been reported to ameliorate human intestinal inflammation in patients with inflammatory bowel disease (IBD).[1] The genomic DNA, double -stranded RNA, and cell wall components of lactobacilli have been identified as components responsible for the anti-inflammatory effects.[2-5] Specifically, there have been a number of reports regarding the anti-inflammatory effects of genomic DNA from lactobacilli.[5-9] Rachmilewitz et al. reported that intragastric administration of probiotic medical food VSL#3® (VSL Pharmaceuticals, Inc.), which consists of eight strains of lactic acid bacteria including lactobacilli, ameliorated the severity of colitis in a dextran sodium sulfate (DSS)-induced mouse model of IBD, whereas DNase-treated VSL#3® had no effect.[5] They also demonstrated that VSL#3® had no effect on the severity of DSS-induced colitis in mice deficient for

toll-like receptor 9 (TLR9), which is a specific receptor of bacterial DNA[10] and has been reported to play a role in one of the major pathways responsible for the anti-inflammatory effects of genomic DNA from lactobacilli.[6,8] Taken together, these observations suggest that the anti-inflammatory effects of lactobacilli are mediated by their genomic DNA, and TLR9 signaling is essential for these anti-inflammatory effects.

IBD, including Crohn's disease and ulcerative colitis, are chronic inflammatory disorders of the intestine. Although the etiology of IBD has yet to be determined, a number of mediators seem to play prominent roles in the pathogenesis of chronic inflammatory disorders. Recent studies have highlighted the roles of proteases and their endogenous inhibitors in the pathogenesis of enteritis.[11-14] Elevated proteolytic activities of several proteases have been detected in the intestinal tissues of IBD patients.[11] This increased proteolytic activity may result from decreased efficacy and expression of endogenous protease inhibitors.[12] These results suggest that the shift of protease/anti-protease balance toward a more proteolytic environment causes chronic inflammation in the intestine. Among the endogenous protease inhibitors, elafin is a natural inhibitor of the human neutrophil proteases, elastase and proteinase 3.[15,16] Elafin is mainly secreted from the epithelial cells of various tissues, including the intestine,[17] and exerts anti-inflammatory effects by protease inhibition and by restoring barrier function to damaged epithelial cells in the intestine.[13] The expression of elafin was downregulated in the intestinal tissues of IBD patients, and this was associated with increased elastase activity.[14] In addition, a previous study using transgenic mice expressing human elafin demonstrated that an increased level of human elafin in the intestine re-equilibrates the protease/anti-protease balance and prevents intestinal inflammation in DSS- and trinitrobenzene sulfonic acid (TNBS)-induced mouse models of IBD.[13] These observations suggest that an increased level of elafin in the intestine may be useful in the treatment of IBD.

In the present study, we hypothesized that lactobacilli may increase elafin secretion from intestinal epithelial cells, as both lactobacilli and increased levels of elafin are involved in intestinal anti-inflammatory effects. In this context, Motta et al. engineered recombinant human elafin-expressing lactic acid bacteria, including lactobacilli, and demonstrated that oral administration of these recombinant bacteria ameliorates the symptoms of inflammation in DSS-induced murine colitis.[14] However, the increase in elafin by lactobacilli in the intestine has not been characterized in rodent studies, because a rodent homolog of human elafin has not been identified. Therefore, we examined elafin secretion from the human epithelial colorectal adenocarcinoma cell line, Caco-2, a model of human intestinal epithelial cells, induced by *Lactobacillus plantarum*, which is widely used in the food industry due to its metabolic versatility, and it has been reported to show anti-inflammatory effects both *in vitro* and *in vivo*.[18,19] To elucidate the molecular basis underlying the upregulation of elafin secretion by *L. plantarum*, we examined the roles of the bacterial genomic DNA and TLR9 in this effect.

Methods

Bacterial strain and culture conditions

L. plantarum strain D2905 was used in this study.[20] The bacterium was grown at 27 °C, the optimal temperature for its culture, in MRS medium under static conditions. To produce heat-killed *L. plantarum*, an overnight culture was inoculated into MRS medium to an optical density at 600 nm (OD_{600}) of 0.05 and grown for 8 h. After centrifugation (7,000 × *g*, 15 min, 4 °C) and two washes with PBS, the bacterium was incubated for 10 min at 65 °C. The bacterium was killed by this incubation (bacterial viability: 0.001%). This heat-killed bacterial preparation was used without centrifugation and washing in the following experiments. Furthermore, DNase-treated heat-killed *L. plantarum* was prepared by incubation with 5 μg/mL DNase I (Roche) for 16 h at 37 °C.

Preparation of genomic DNA from L. plantarum

Genomic DNA was extracted from an overnight culture of *L. plantarum* using a Gentra Puregene Yeast/Bact. Kit (Qiagen) according to the manufacturer's protocol. The concentration and purity of genomic DNA preparation were confirmed by measuring OD_{260}, and $OD_{260/280}$ and $OD_{260/230}$ ratios, respectively. Only genomic DNA with $OD_{260/280} > 1.8$ and $OD_{260/230} > 2.0$ was used for the experiments. Purified genomic DNA was tested for endotoxin contamination using Limulus Amebocyte Lysate QCL-1000 (Lonza).

Measurement of elafin secretion by L. plantarum

Caco-2 cells were used as described previously.[6] Aliquots of 2×10^5 Caco-2 cells were plated in each well of 24-well plates (Nunc). Cells were treated with three concentrations of live or heat-killed *L. plantarum* (1, 2, and 5×10^9 CFU/mL) for 12, 24, and 48 h. After incubation, cell supernatants were centrifuged at 15,000 × *g* for 5 min at 4 °C. The concentration of elafin in the cell supernatants was determined using a human elafin ELISA kit (R & D Systems).

Measurement of elafin secretion by transfected L. plantarum genomic DNA

Aliquots of 2×10^5 Caco-2 cells were plated in each well of 24-well plates. Subsequently, 5 μL of *L. plantarum* genomic DNA (0.35, 0.7, and 1.7 mg/mL) was added to 245 μL of minimum essential medium (Invitrogen) containing 1% fetal bovine serum (Biowest). The genomic DNA solution was then added to 250 μL of medium containing 1.25 μL of siLentFect Lipid Reagent (Bio-Rad Laboratories) and incubated for 30 min at room temperature to produce the transfection mix. Caco-2 cells were transfected with the transfection mix for 12, 24, and 48 h. After incubation, elafin secretion was measured by ELISA as described above. As the genome size of *L. plantarum* is approximately 3 Mbp, 1, 2, and 5×10^9 CFU/mL of *L. plantarum* contain approximately 3.5, 7, and 17 μg/mL of genomic DNA, respectively. Therefore, we used these three concentrations of genomic DNA (3.5, 7, and 17 μg/mL) in this experiment.

Transfection of Caco-2 cells with TLR9-siRNA

TLR9 expression in Caco-2 cells was suppressed by RNAi, as described previously.[21] Briefly, aliquots of 2×10^5 Caco-2 cells plated in 24-well plates were transfected with 50 nM TLR9

-siRNA or scramble TLR9-siRNA (Bonac Corp.) as a negative control using siLentFect Lipid Reagent. After incubation for 24 h, the cells were treated with heat-killed *L. plantarum* (5×10^9 CFU/ml) or transfected with *L. plantarum* genomic DNA (17 μg/mL) for 48 h as described above. The cell supernatants were collected, and the cellular protein extracts from Caco-2 cells were prepared with PRO-PREP Protein Extraction Solution (iNtRON Biotechnology) according to the manufacturer's protocol. The concentration of elafin in the cell supernatants was determined by ELISA. Suppression of TLR9 expression in the protein extracts of cells transfected with TLR9-siRNA, but not scramble TLR9-siRNA, was confirmed by Western blotting using an antibody to TLR9 as described previously.[6]

Detection of intracellular translocated genomic DNA

Aliquots of 2×10^5 Caco-2 cells were plated in each well of 24-well plates and treated with heat-killed *L. plantarum* (5×10^9 CFU/ml) or *L. plantarum* genomic DNA (17 μg/mL) for various times (0, 0.5, 1, 3, 6, 24, and 48 h). After treatment, Caco-2 cells were trypsinized using trypsin-EDTA (Invitrogen) at 37 °C for 10 min. The cells were then collected and centrifuged at 1,000 × *g* for 5 min and washed five times with PBS. DNA was extracted from Caco-2 cells using ISOGEN (Nippon Gene) according to the manufacturer's protocol. Detection of intracellular genomic DNA was performed by PCR based on the *L. plantarum*-specific 16S rRNA gene.[6] PCR was performed using KOD FX Neo (Toyobo) in 30-μL reaction mixtures containing 1 μg of DNA preparation and the following primers: sense 5'-TGGTATTGATTGGTGCTTGCA-3' and anti-sense 5'-CCACCTTCCTCCGGTTTGTCA-3'. PCR products were subjected to 1.5% agarose gel electrophoresis.

Statistical analysis

Data are presented as means ± standard deviations. Statistical analyses were performed using Origin Pro 8.1 (OriginLab). In all analyses, $P < 0.05$ was taken to indicate significance.

Results

Increase in elafin secretion by heat-killed L. plantarum

Treatment with heat-killed *L. plantarum* (5×10^9 CFU/ml) significantly increased elafin secretion from Caco-2 cells, reaching 0.84 ng/mL after 48 h of treatment ($P < 0.01$ vs. medium alone by two-way ANOVA followed by Tukey's test) (**Figure 1A**). In contrast, elafin secretion remained close to the background level after 48 h of treatment with live *L. plantarum*. Therefore, heat-killed *L. plantarum* was used in the following experiments. As shown in **Figure 1B**, treatment for 48 h with heat-killed *L. plantarum* at 2 and 5×10^9 CFU/mL significantly increased elafin secretion from Caco-2 cells compared to medium alone (0.38 and 0.84 ng/mL vs. 0.10 ng/mL, respectively; $P < 0.01$, one-way ANOVA followed by Tukey's test). In contrast, there was no significant difference in elafin secretion between cells treated with 1×10^9 CFU/mL heat-killed *L. plantarum* and medium alone. These results indicated that heat-killed *L. plantarum* increased elafin secretion in a time- and dose-dependent manner.

Decrease in heat-killed L. plantarum-induced elafin secretion by DNase treatment

Treatment with DNase-treated and non-treated heat-killed *L. plantarum* significantly increased the level of elafin secretion from Caco-2 cells compared with medium alone (1.18 and 0.54 ng/mL vs. 0.20 ng/mL, respectively; $P < 0.01$, one-way ANOVA followed by Tukey's test). However, the level of elafin secretion in cells treated with DNase-treated heat-killed *L. plantarum* was significantly lower than that in cells treated with non-treated heat-killed *L. plantarum* ($P < 0.05$) (**Figure 2**). These observations indicated that DNase treatment partially abolished the increase in elafin secretion by heat-killed *L. plantarum*, although we could not exclude the possibility that DNase caused the decrease in elafin secretion by heat-killed *L. plantarum* regardless of the DNase activity.

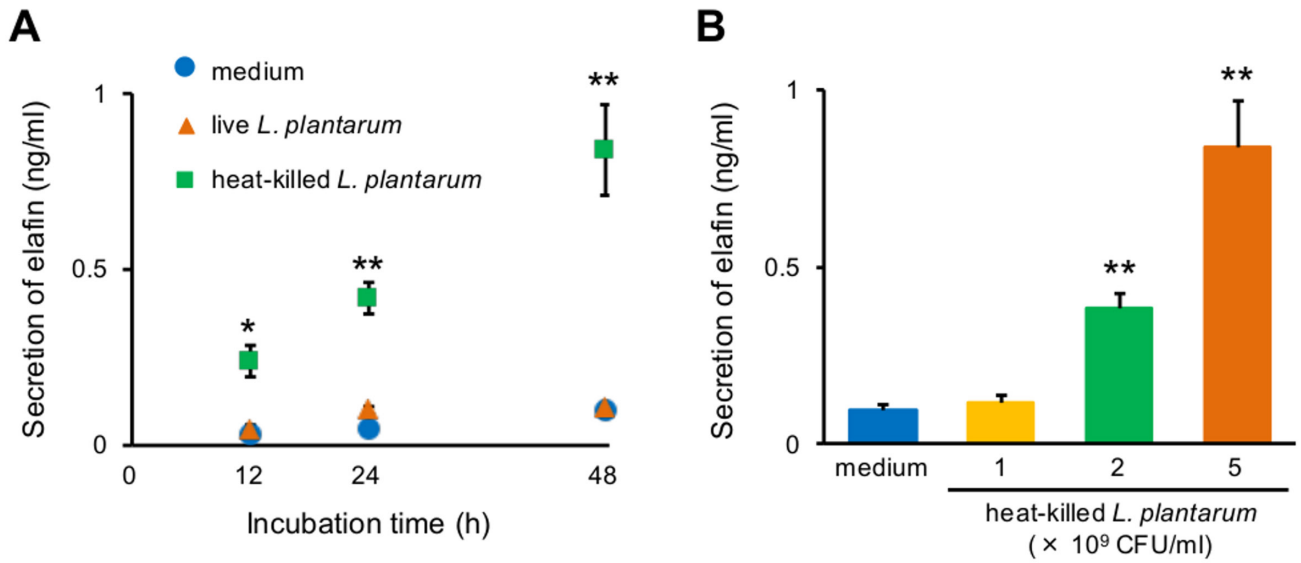

Figure 1. Time- and dose-dependent increases in elafin secretion by heat-killed *Lactobacillus plantarum*.
Caco-2 cells were treated with live or heat-killed *L. plantarum* (5×10^9 CFU/ml) for 12, 24, and 48 h (A), or with three concentrations of the heat-killed *L. plantarum* (1, 2, and 5×10^9 CFU/ml) for 48 h (B). The elafin concentrations in the cell supernatants were determined by ELISA. Data are expressed as ng/ml and represent the means ± standard deviations of three separate experiments performed in triplicate. *$P < 0.05$, **$P < 0.01$ compared with medium alone.

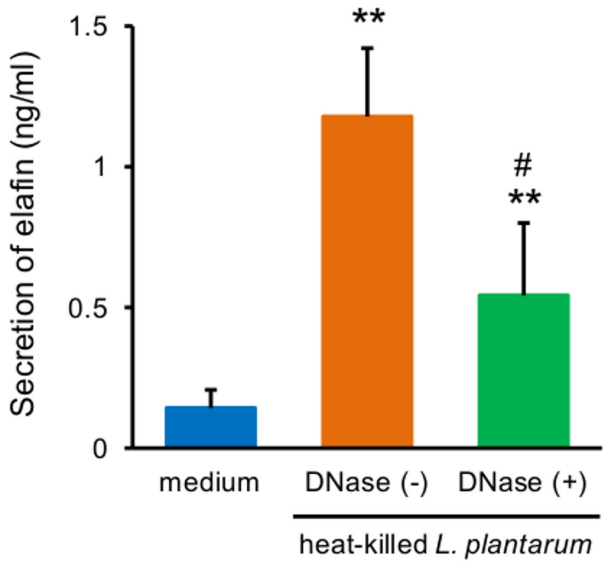

Figure 2. Influence of DNase treatment on elafin secretion by heat-killed *Lactobacillus plantarum*.
Caco-2 cells were treated with DNase-treated (DNase (+)) or non-treated (DNase (–)) heat-killed *L. plantarum* (5×10^9 CFU/ml) for 48 h. The elafin concentrations in the cell supernatants were determined by ELISA. Data are expressed as ng/ml and represent the means ± standard deviations of three separate experiments performed in triplicate. $^{**}P < 0.01$ compared with medium alone, $\#P < 0.05$ compared with DNase non-treated heat-killed *L. plantarum*.

Increase in elafin secretion by L. plantarum genomic DNA

Transfection with *L. plantarum* genomic DNA (17 μg/mL) significantly increased elafin secretion from Caco-2 cells, reaching 0.33 ng/mL after 48 h of treatment ($P < 0.01$ vs. medium alone, two-way ANOVA followed by Tukey's test) (**Figure 3A**). As shown in **Figure 3B**, transfection with *L. plantarum* genomic DNA at 7 and 17 μg/mL for 48 h significantly increased elafin secretion from Caco-2 cells compared with medium alone (0.17 and 0.33 ng/mL vs. 0.07 ng/ml, respectively; $P < 0.01$, one-way ANOVA followed by Tukey's test). In contrast, no significant change in elafin secretion was observed in those transfected with 3.5 μg/mL of genomic DNA compared to controls. These results indicated that *L. plantarum* genomic DNA increased elafin secretion in a time- and dose-dependent manner.

Mediation of TLR9 on elafin secretion by heat-killed L. plantarum and its genomic DNA

TLR9-siRNA-transfected Caco-2 cells showed decreased TLR9 expression compared to siRNA non-transfected and scramble TLR9-siRNA-transfected cells as a negative control (**Figure 4A**). This observation confirmed that TLR9-siRNA suppressed TLR9 expression in Caco-2 cells. Treatment with heat-killed *L. plantarum* significantly increased the level of elafin secretion in non-transfected cells, scramble TLR9-siRNA -transfected cells, and TLR9-siRNA-transfected cells (0.74, 0.80, and 0.39 ng/mL, respectively). On the other hand, treatment with *L. plantarum* genomic DNA significantly increased elafin secretion in non-transfected cells and scramble TLR9-siRNA -transfected cells (0.33 and 0.35 ng/mL, respectively), but the genomic DNA had no such effect in TLR9-siRNA-transfected cells (0.09 ng/mL). In addition, the levels of elafin secretion in TLR9-siRNA-transfected cells treated with heat-killed *L. plantarum* and *L. plantarum* genomic DNA were significantly lower than those in non-transfected cells and scramble TLR9 -siRNA-transfected cells ($P < 0.05$ and $P < 0.01$, respectively; two-way ANOVA followed by Tukey's test) (**Figure 4B**). These observations indicated that suppression of TLR9 expression partially or completely abolished elafin secretion by heat-killed *L. plantarum* and its genomic DNA, respectively.

Intracellular translocation of L. plantarum genomic DNA

Intracellular translocation of the genomic DNA was a prerequisite for recognition by TLR9, because TLR9 is localized in

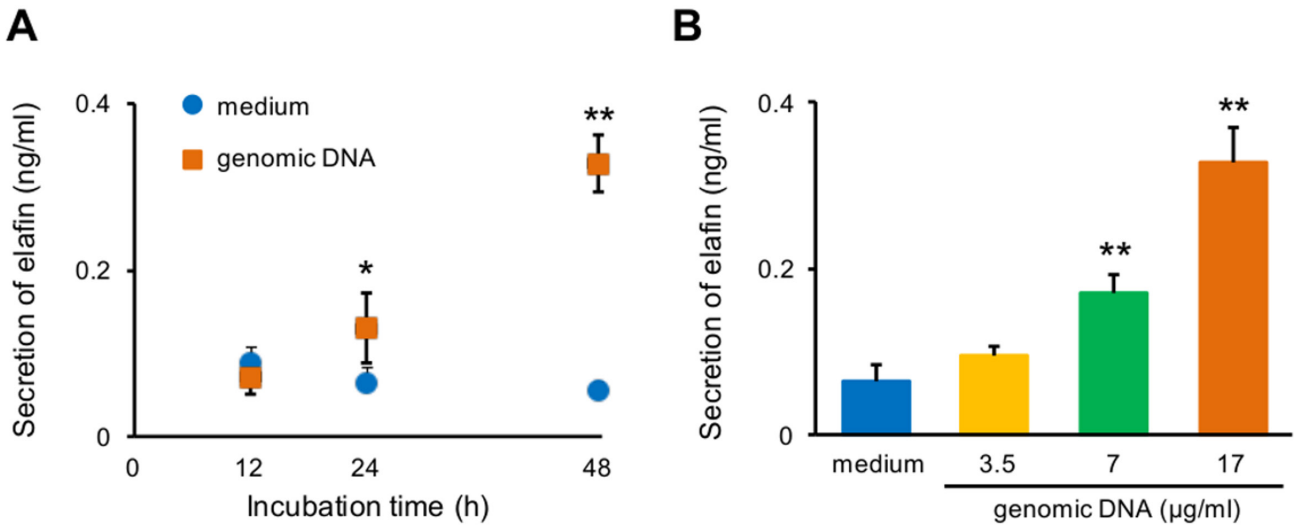

Figure 3. Time- and dose-dependent elafin secretion by genomic DNA from *Lactobacillus plantarum*.
Caco-2 cells were transfected with *L. plantarum* genomic DNA (17 μg/ml) for 12, 24, or 48 h (A) or with three concentrations of the genomic DNA (3.5, 7, and 17 μg/ml) for 48 h (B). The elafin concentrations in the cell supernatants were determined by ELISA. Data are expressed as ng/ml and represent the means ± standard deviations of three separate experiments performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with medium alone.

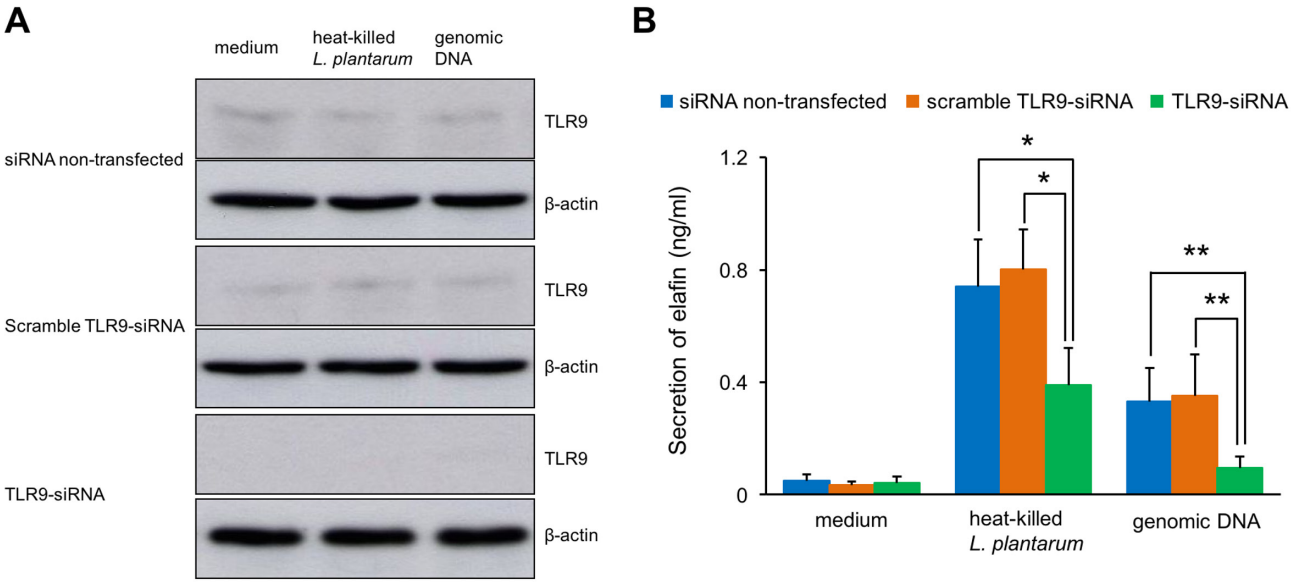

Figure 4. Effects of TLR9 suppression on elafin secretion by heat-killed *Lactobacillus plantarum* and its genomic DNA.
TLR9-siRNA- and scramble TLR9-siRNA-transfected Caco-2 cells were treated with heat-killed *L. plantarum* (5×10^9 CFU/ml) or transfected with *L. plantarum* genomic DNA (17 μg/ml) for 48 h. (A) TLR9 expression in cellular protein extract was confirmed by Western blotting using anti-TLR9 antibody. β-actin was used as an internal control. Data are representative of three separate experiments. (B) The elafin concentrations in the cell supernatants were determined by ELISA. Data are expressed as ng/ml and represent the means ± standard deviations of three separate experiments performed in triplicate. $^*P < 0.05$, $^{**}P < 0.01$.

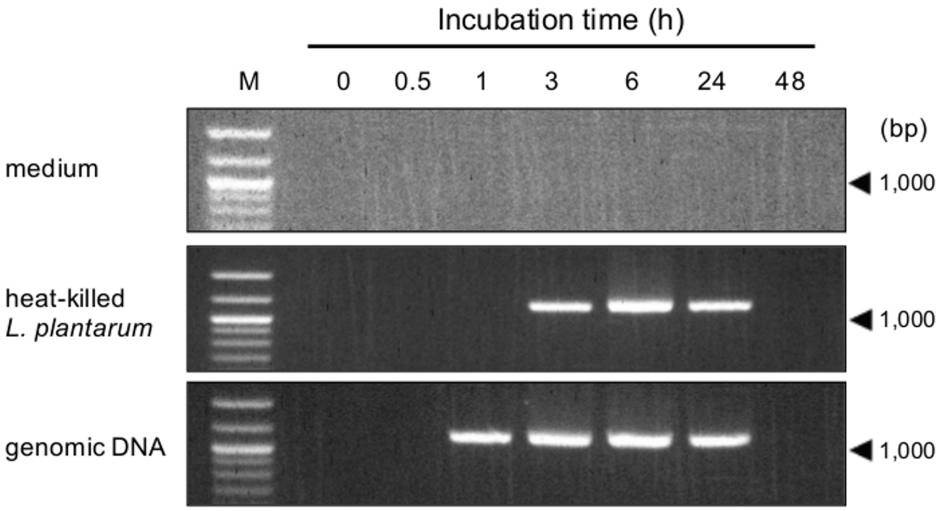

Figure 5. Intracellular translocation of genomic DNA from *Lactobacillus plantarum* in Caco-2 cells.
Caco-2 cells were treated with heat-killed *L. plantarum* (5×10^9 CFU/ml) or *L. plantarum* genomic DNA (17 μg/ml) for various times (0 – 48 h). Intracellular translocation of the genomic DNA was detected by PCR based on the coding sequence of the *L. plantarum*-specific 16S rRNA gene. Data are representative of three separate experiments. M; 100 bp DNA ladder marker.

the endosome and lysosome.[22] Therefore, we examined whether *L. plantarum* genomic DNA can be translocated into Caco-2 cells. *L. plantarum* genomic DNA was detected within Caco-2 cells at 3–24 h after treatment with heat-killed *L. plantarum*, and the genomic DNA was also detected within cells at 1–24 h after treatment (**Figure 5**). No *L. plantarum* genomic DNA was detected within cells following treatment with medium alone.

Discussion

The results of the present study indicated that treatment with heat-killed *L. plantarum* increased the level of elafin secretion from Caco-2 cells, which was partially dependent on the bacterial genomic DNA. Moreover, TLR9 was shown to be the primary mediator of elafin secretion by heat-killed *L. plantarum* and its genomic DNA. To our knowledge, this is the first report regarding induction of elafin secretion from intestinal epithelial cells by lactobacilli and the molecular mechanisms underlying this effect.

Over the past several decades, lactobacilli have been reported to show anti-inflammatory effects and have been used

to treat patients with IBD.[1] Increased elafin levels have been shown to play an important role in intestinal anti-inflammatory effects.[13,14] However, there have been no previous reports of increases in elafin levels induced by lactobacilli in the intestine. As elafin production has not been characterized in rodents, we examined whether lactobacilli could increase the level of elafin in the intestine using Caco-2 cells, and our results indicated that heat-killed *L. plantarum* increased elafin secretion from Caco-2 cells (**Figure 1**). These observations suggested that orally administered lactobacilli may increase elafin levels in the intestines of IBD patients. As the protease/anti-protease imbalance due to downregulation of elafin was shown to be involved in chronic inflammation in the intestine of IBD patients,[11-14] the increase in elafin levels by lactobacilli in the intestine may lead to re-equilibration of the protease/anti-protease balance and subsequently ameliorate inflammation in these patients. On the other hand, live *L. plantarum* had no effect on elafin secretion (**Figure 1**), although live lactobacilli therapy has been reported to ameliorate human IBD.[1] In general, orally administered live lactobacilli cannot survive in the low pH gastric environment, and the secretion of lysozymes, bile acid, and pancreatic fluid in the duodenum also influence the viability of the lactobacilli.[23,24] Consequently, the lactobacilli would be killed by these conditions prior to reaching the intestine. Thus, it is possible that orally administered live lactobacilli are killed, and then increase elafin secretion from human intestinal epithelial cells.

Genomic DNA has been identified as the principle anti-inflammatory component of lactobacilli.[5-9] Therefore, we hypothesized that the increase in elafin secretion by heat-killed *L. plantarum* depends on its genomic DNA. In the present study, DNase treatment partially abolished the increase in elafin secretion by heat-killed *L. plantarum* (**Figure 2**). Moreover, *L. plantarum* genomic DNA increased elafin secretion from Caco-2 cells, although to a lesser extent than heat-killed *L. plantarum* (**Figures 1 and 3**). These results indicated that the increase in elafin secretion by *L. plantarum* is partially mediated by its own genomic DNA and suggested that lactobacilli may exert anti-inflammatory effects through genomic DNA-dependent elafin secretion from epithelial cells in the human intestine. These observations also suggested that bacterial components other than genomic DNA may also be involved in the increase in elafin secretion by heat-killed *L. plantarum*. Previous studies showed that dsRNA and cell wall components are also anti-inflammatory components of lactobacilli.[2-4] Therefore, the increase in elafin secretion by heat-killed *L. plantarum* may be related not only genomic DNA but also to these other bacterial components.

The anti-inflammatory effects of lactobacilli genomic DNA are primarily mediated by TLR9 signaling.[5,6] Consistent with this, suppression of TLR9 completely abolished the increase in elafin secretion by *L. plantarum* genomic DNA in the present study (**Figure 4**). These observations indicated that TLR9 signaling is a major pathway responsible for the induction of elafin secretion by *L. plantarum* genomic DNA. However, suppression of TLR9 only partially abolished the increase in elafin secretion by heat-killed *L. plantarum*, suggesting that TLR9 is one, but not the only, mediator of elafin secretion by heat-killed *L. plantarum*. As bacterial components other than genomic DNA, including dsRNA and cell wall components, may be involved in the upregulation of elafin secretion by heat-killed *L. plantarum*, the receptors for these bacterial components (i.e., TLR3 and TLR2)[25,26] may also partially regulate these effects. Further studies are required to fully elucidate the molecular mechanism of elafin secretion by heat-killed *L. plantarum*.

Overproduction of inflammatory cytokines results in the development of inflammation in the intestine. In fact, the levels of the inflammatory cytokine interleukin (IL)-8 have been shown to be increased in the intestine of IBD patients.[27] Therefore, it is important to examine IL-8 secretion in assessment of the level of inflammation in the intestine. Our previous study indicated that genomic DNA of lactobacilli decreased H_2O_2-induced IL-8 secretion from Caco-2 cells.[6] In addition, we identified an oligodeoxynucleotide (ODN), which markedly decreased H_2O_2-induced IL-8 secretion, from the genomic DNA of *L. casei*, and demonstrated that oral administration of this anti-inflammatory ODN to mice ameliorated DSS-induced murine colitis.[21] Therefore, suppression of IL-8 secretion *in vitro* is an important factor for ameliorating colitis in this *in vivo* model of IBD. In our preliminary study, we found that heat-killed *L. plantarum* and its genomic DNA decreased H_2O_2-induced IL-8 secretion from Caco-2 cells without inducing a pro-inflammatory response (data not shown). These observations suggested the possibility of an anti-inflammatory effect in an *in vivo* model of IBD by heat-killed *L. plantarum* and its genomic DNA. On the other hand, we also showed previously that suppression of TLR9 expression by RNAi abolished the decrease in H_2O_2-induced IL-8 secretion by lactobacilli genomic DNA and the anti-inflammatory ODN.[6,21] These observations indicated that the TLR9 signaling pathway plays a major role in mediating the decrease in IL-8 secretion. However, at present, we cannot explain the mechanism by which activation of the TLR9 signaling pathway results in the observed decrease in IL-8 secretion. The results of the present study indicated that *L. plantarum* genomic DNA increased elafin secretion from Caco-2 cells through TLR9 (**Figures 3 and 4**). Therefore, the increase in elafin levels may play important roles in the reduction of IL-8 secretion by lactobacilli genomic DNA, likely due to inhibition of pro-inflammatory proteases and restoration of barrier function to damaged epithelial cells.[13] Further studies of the increase in elafin levels may elucidate the mechanism by which the TLR9 signaling pathway induces the observed anti-inflammatory effects.

TLR9 is expressed in human epithelial cells, including Caco-2 cells, and is localized in the endosome and lysosome.[22] The bacterial genomic DNA must undergo translocation into Caco-2 cells to be recognized by TLR9. However, bacterial genomic DNA cannot translocate into mammalian cells because of its high molecular weight and hydrophilicity. Therefore, translocation of DNA into eukaryotic cells has generally been performed using transfection reagents, such as liposomes. In the present study, we confirmed that *L. plantarum* genomic DNA translocated into Caco-2 cells in the presence (**Figure 5**), but not in the absence (data not shown), of transfection reagent. However, translocation of genomic DNA was also observed in Caco-2 cells treated with heat-killed *L. plantarum*. At present, we cannot explain these observations. Previous

studies showed that *L. plantarum* produces a major polyamine, putrescine,[28] and that polyamines form a complex with and stabilize the DNA. This complex subsequently forms nanoparticles and is subsequently readily translocated into eukaryotic cells.[29,30] One possible explanation for the translocation of genomic DNA into Caco-2 cells treated with heat-killed *L. plantarum* is that the genomic DNA and polyamine contained in heat-killed *L. plantarum* may form such a complex, thus facilitating translocation of the genomic DNA into Caco-2 cells due to the transfection effect of the polyamine.

Conclusion

The results of the present study indicated that heat-killed *L. plantarum* increased the level of elafin secretion from Caco-2 cells, and the bacterial genomic DNA and TLR9 play crucial roles in this effect. Lactobacilli therapy is useful for the treatment of IBD, although the underlying mechanisms are not fully understood. Our observations suggest that one possible mechanism for the efficacy of lactobacilli therapy may involve an increase in elafin levels in the intestine. Further studies of the elafin induction associated with lactobacilli and their genomic DNA may lead to the development of novel therapeutic approaches to ameliorate intestinal inflammation.

Acknowledgement

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ORIGINAL ARTICLE

Common antibody dependent cell mediated cytotoxicity (ADCC) antibody epitopes of HIV-1 CRF01_AE Env and Gag in early HIV-1 infected individuals

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Abstract

Background: There have been a few studies aimed at identifying epitopes of ADCC-inducing antibodies when compared to those of neutralizing antibodies and cytotoxic T lymphocytes against a variety of HIV-1 clades.

Objective: To map the common ADCC epitopes of HIV-1 CRF01_AE.

Methods: We screened 65 sera of confirmed early HIV-1 CRF01_AE infected individuals for ADCC antibody against gp120 utilizing an EGFP-CEM-NK^r flow cytometric assay. Sera with high ADCC antibody were then examined against ADCC epitopes using the complete HIV-1 CRF01_AE gp160- and subtype A Gag-overlapping peptide sets which were divided into 7 pools:E1-E7 and 5 pools:G1-G5, respectively. Each positive peptide pool was further investigated for fine ADCC epitope mapping using matrix formats.

Results: Twenty, 25 and 20 sera demonstrated the high-, medium- and low-ADCC antibody activities against gp120, respectively. Interestingly, 11 Env- and 6 Gag-peptides of pools E3, E4, E7 and pools G1, G2, G4 with high ADCC responses were also responded by at least 20%, 12% and 5%, 10% of medium- and low-ADCC antibody sera, respectively. These eleven common Env ADCC epitopes were localized at C2-V3-C3-V4 regions of gp120 and cytoplasmic tail of gp41 while six common Gag ADCC epitopes were localized at p17-p24-p2 regions.

Conclusions: Although the degree of ADCC antibody responses to the gp120 protein varied from high to low, there were certain consensus Env and Gag peptides that could induce the ADCC antibody responses of 21.54-58.46% and 23.08-41.54%, respectively of the early infected individuals. This epitope information should be useful as the new antibody-based vaccine immunogens.

Key words: ADCC antibody epitopes; HIV-1 CRF01_AE; Env; Gag; early infection

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Introduction

The development of effective HIV-1 vaccines is urgently required for controlling future spread of HIV-1.[1] Current prophylaxis HIV-1 vaccine trials are based on both arms of immune responses, i.e., humoral and cellular immunity. However, each arm has not yet been successful.[2] Antibody dependent cell mediated cytotoxicity (ADCC) is a cytolytic mechanism of natural killer (NK) cells against virus-infected cells by combining of the innate and adaptive immunity. The mechanism of ADCC can be stimulated by the interaction of specific antibody bound to HIV-1 antigens on the infected cells and Fc receptor CD16 (FcγRIIIa) expressed on the surface of NK cells. Activation of NK cells lead to lysis of target cells.[3]

ADCC responses have been reported to correlate with the control or limit viremia during acute HIV-1 infection and help to slow the progression of disease in both HIV-1 infected individuals and SIV infected macaques.[4,5] Of the six HIV-1 efficacy trials, the RV144 prime-boost regimen phase III trial has provided evidence of vaccine-induced 31.2% protection.[2] Vaccinees who showed significantly low risk of HIV-1 infection demonstrated the high level of ADCC antibody responses.[6]

In the clinical course of HIV-1 infection, the acute phase of infection reveals an initial high peak of plasma viremia and decreased number of $CD4^+$ T lymphocytes.[7] In addition, expansion of inefficient HIV-1-specific $CD8^+$ T cells during acute infection has recently been reported.[8] Finding of the early effective immune responses against transmitted virus in acute HIV-1 infection may give a chance to control initial viremia, prolong HIV-1 disease progression, decrease immune cell damage and prevent transmission.[9] Identification of the common epitopes of HIV-1 that could respond to ADCC antibody of sera with low-, medium- or high-ADCC against envelope protein at the early phase would be an important new information that can be utilized for vaccine development. Here we have identified common ADCC antibody epitopes of Env and Gag of HIV-1 CRF01_AE using sera of confirmed early HIV-1 infected individuals.

Methods

Volunteer samples

All sera samples from young Thai men who were enrolled to the conscripts during year 2001-2013 were obtained with written informed consents following approval from Institutional Review Board (IRB) of Royal Thai Army Medical Department (Code: S034b/57) and IRB of the Faculty of Medicine Siriraj Hospital (COA: Si609/2014) prior to initiation of this study. The sera samples obtained had no individual identifiers. These sera were confirmed as early HIV-1 infection by using the Aware™ BED™ EIA HIV-1 Incidence Test (Calypte Co., Portland, OR, USA).

Cell line

The NK cell resistant, EGFP-CEM-NK^r cells were cultured and prepared for the assay as previously described.[10]

Peptides

The gp120 of HIV-1 CRF01_AE CM243 (Protein Sciences, Meriden, CT, USA) was used to pulse the EGFP-CEM-NK^r cells. In addition, the complete peptide set based on the HIV-1 CRF01_AE Env TH023/CM240 sequence consists of 168 peptides (122 peptides of TH023 gp120 and 46 peptides of TH023/CM240 gp41) each containing 15 amino acids (aa) in length, overlapping by 11 aa. The 143 peptides of TH023 were kindly provided by the Natural and Medical Sciences Institute (University of TÜbingen, Germany) and 25 peptides of CM240 were commercially synthesized (GenScript, Piscataway, NJ, USA). These Env peptides were divided into seven pools:E1-E7, each containing 24 peptides. To further identify the reactive peptide, each informative peptide pool was again divided into ten small pools according to the 5 by 5 matrix formats. Moreover, Gag peptide set of HIV-1 subtype A sequence consisting of 49 peptides each containing 20 aa in length, overlapping by 10 aa was kindly provided by the National Institutes of Health (NIH) AIDS reagent repository (catalog #3991). These Gag peptides were divided into five pools:G1-G5, each containing 9-10 peptides. Each pool was again divided into three small pools as 3 by 3 matrix formats. Each peptide pool was also used to pulse the EGFP-CEM-NK^r cells.

Preparation of sera

All sera were from individuals infected with HIV-1 CRF01 _AE as determined by C2-V4 nucleotide sequencing as described elsewhere.[11] A HIV-1 sero-negative pooled serum and a sero-positive serum with high ADCC antibody activity were used as controls with each assay. All sera were inactivated at 56 °C for 30 min. prior to use and used at dilutions of 1:250, 1:2,500 and 1:25,000.

Target cells

The gp120- or peptide pool-pulsed EGFP-CEM-NK^r target cells were prepared by the addition of either 0.1 μg gp120 of HIV-1 CRF01_AE CM243 or 1 μg of each peptide pool to 1×10^5 target cells. The peptide and target cells were incubated for 1 h at room temperature. The gp120 pulsed cells were washed three times with phosphate buffered saline. The target cells were then re-suspended in growth medium containing RPMI 1640 (Gibco, Grand Island, NY) supplemented with 2 mM l-glutamine, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 10% heat-inactivated fetal calf serum (Gibco) at a concentration of 1×10^5 cells/ml.

Effector cells

Cryopreserved peripheral blood mononuclear cells (PBMC) were prepared from leukopaks of a single HIV-1 seronegative blood donor as previously described.[10] Cells were later thawed and overlaid overnight at 1×10^6 cells/ml in growth medium at 37 °C with 5% CO2. The cells were then re-suspended in growth media at a concentration of 2.5×10^6 cells/ml and used as effector cells.

EGFP-ADCC flow cytometric assay

The EGFP-ADCC flow cytometric assay was based on a method previously described.[10] Lysis of target cells by ADCC activity was measured and analyzed with a FACScan flow cytometer (Becton Dickinson Biosciences {BDB}, San Jose, CA, USA). The actual percent lysis of target cells at each serum dilution was calculated as previously reported.[10] The cut-off for positive results using gp120- and peptide pool-pulsed target cells are 7.21% lysis and 5.46% lysis, respectively.[12]

Results

Screening for high ADCC antibody activity against gp120 pulsed target cells

Sixty five confirmed early HIV-1 CRF01_AE infected sera were investigated for ADCC antibody activity against gp120 pulsed target cells. The percent lysis of target cells ranged from 9.15 % to 56.12 % with mean and SD values of 24.08 and 10.72, respectively. Twenty, 25 and 20 sera demonstrated the high (> 29.44% target cell lysis, > mean + 0.5SD), medium

(29.44-18.72% lysis, mean ± 0.5SD) and low (< 18.72% lysis, < mean-0.5SD) ADCC antibody activities, respectively.

Identification of HIV-1 CRF01_AE gp160- and Gag-epitopes that are the target of ADCC reactive antibodies

Twenty sera with relatively high ADCC antibody activity were further examined against each of the seven peptide pools:E1-E7 of the HIV-1 CRF01_AE Env peptide set and each of the five peptide pools:G1-G5 of HIV-1 subtype A Gag formulated as described in the methods section. These 20 sera demonstrated positive ADCC antibody responses against a number of Env peptide pools while 18 sera responded against Gag peptide pools as shown in **Table 1**. For Env peptide pools, whereas sixteen (80%), fourteen (70%), and twelve (60%) sera demonstrated significantly higher frequency of ADCC responses against pools E7, E3 and E4, respectively, six to ten (30-50%) of these sera showed significant but somewhat lower frequency of ADCC responses to peptide pools E2, E1, E6 and E5. Moreover, 9/20 (45%) of tested sera demonstrated the responses to all three peptide pools: E3, E4, E7. In addition, all tested sera responded to at least one of these three peptide pools. Thus, a 5 by 5 matrix of each of the 3 peptide pools E3, E4 and E7 was selected to perform the fine epitope mapping of ADCC responses. The potential ADCC antibody epitopes were identified by verifying the common peptides from this matrix. The common peptides within pools E3, E4 and E7 that responded to ADCC activity of each of the 20 tested sera are shown in **Table 2**. Twelve potential ADCC antibody epitopes with ≥ 50% responses of all tested sera in each pool were identified. For the parent E3 peptide pool, 9/14 (64.29%) and 7/14 (50%) sera showed ADCC activity against peptides (78, 83) and 71, respectively. For the parent E4 pool, 8/12 (66.67%), 7/12 (58.33%) and 6/12 (50%) of sera showed ADCC activity against peptides 87, (84, 85, 95, 97) and (89, 92), respectively. Moreover, 8/16 (50.00%) sera demonstrated ADCC activity against peptides 190 and 191 from the parent E7 pool.

For Gag peptide pools, whereas fourteen (70%), twelve (60%) and ten (50%) sera demonstrated significantly higher frequency of ADCC antibody responses against pools G4, G2 and G1, respectively, three to seven (15-35%) of these sera showed significant but somewhat lower frequency of ADCC responses to pools G3 and G5 (**Table 1**). In addition, 6/20 (30%) of tested sera recognized all three peptide pools: G1, G2, G4. However, two sera (10%) did not respond to any pool of Gag peptide. Thus, a 3 by 3 matrix of each of the 3 peptide pools G1, G2 and G4 was selected to perform the fine epitope mapping of ADCC responses. The common peptides within pools G1, G2 and G4 that responded to ADCC activity of each of the 18 sera tested are shown in **Table 3**. Six potential ADCC antibody epitopes with ≥ 50% responses of all tested sera in each pool

Table 1. Identification of peptide pools that are the targets of antibody dependent cell mediated cytotoxicity (ADCC) antibody responses in high ADCC antibody activity sera.

Serum No.	Obtained year	# Env peptide pools (E1-E7) response to ADCC antibody							# Gag peptide pools (G1-G5) response to ADCC antibody				
11	2001			3							-		
4	2007	1	2			5	6	7	1			4	
14	2007	1	2					7		2		4	5
10	2008	1					6	7				4	
15	2009	1		3	4	5		7		2	3	4	
3	2011			3			6				-		
11	2011	1	2	3		5		7	1	2		4	5
15	2011			3	4			7		2			5
16	2011			3	4			7	1	2			5
4	2012		2		4	5		7	1	2	3	4	5
5	2012			3	4	5	6	7		2			5
7	2012			3	4	5	6	7	1	2		4	
9	2012				4			7	1	2	3		
12	2012			3		5			1	2		4	
17	2012	1		3	4		6	7	1	2		4	
5	2013			3				7				4	
6	2013		2	3	4	5	6	7				4	
7	2013		2		4	5	6					4	5
8	2013	1		3	4	5		7	1	2		4	
9	2013			3	4		6	7	1			4	
Total		**7**	**6**	**14**	**12**	**10**	**9**	**16**	**10**	**12**	**3**	**14**	**7**

High ADCC antibody activity against HIV-1 gp120 pulsed target cells of twenty HIV-1 seropositive sera were analyzed for their individual responses to target cells pulsing with either each of 7 peptide pools (E1-E7) comprising the complete HIV-1 subtype CRF01_AE TH023/CM240 gp160 peptide set consisting of 168 peptides 15 amino acids in length or each of 5 peptide pools (G1-G5) comprising the complete HIV-1 subtype A Gag peptide set consisting of 49 peptides 20 amino acids in length. Each pool of Env- and Gag-peptides contains 24 and 9-10 peptides, respectively. Sera years of HIV-1 infection are also shown.

Table 2. Peptide mapping of high ADCC antibody sera against Env peptide pools E3, E4 and E7.

Serum		Single peptide number of Env pool responses to ADCC antibody			Total peptide responses
No.	Obtained year	Env pool 3 (E3)	Env pool 4 (E4)	Env pool 7 (E7)	
11	2001	**78**, **83**	-	-	2
4	2007	-	-	188, **190**, **191**	3
14	2007	-	-	176, 181, 186	3
10	2008	-	-	175, 176, 180, 181, 185, 186, **190**, **191**	8
15	2009	62, 65, 66, **83**	**87**, 88, **92**, 93, **97**, 98	177, 179, 186-189, **190**, **191**	18
3	2011	**78**	-	-	1
11	2011	70, **71**, 75, 77, **78**, 82, **83**	-	181, **191**	9
15	2011	63, 65, 66, 68, 70, **71**, 75, 77, **78**, 80, 81, **83**	90, **92**, 94, **95**, **97**, 99	179, 180	20
16	2011	70, **71**, 77, **78**, 82, **83**	**84**, **85**, 86, **87**, 88, 90, 91, **92**, 93, **95**, 96, **97**, 98	178-180, 188, 189, **190**	25
4	2012	-	91, **92**, 96, **97**, 101, 102	177, 178, 181, 187, 188, **191**	12
5	2012	56, 57, 61, 62, 66, 67, **71**, 74, **78**, 79, **83**	86, **87**, **89**	168, 173, 178, 180, 188, **190**	20
7	2012	62, 65, 66, 67, 70, **71**, 74, 77, **78**, 79, 82, **83**	**84**, **85**, 86, **87**, 88	168-170, 173-175, 183-185	26
9	2012	-	**84**, **85**, **87**, **89**	172-175, 187-189, **190**	12
12	2012	70, **71**	-	-	2
17	2012	56-59, 62-64, 67-69, **71**	**84**, **85**, **87**, **89**, **95**, **97**, 99, 100, 102, 104	169, 171, 179, 181, 184, 186, 189, **190**, **191**	29
5	2013	**78**	-	**191**	2
6	2013	56, 57, 67, 79	**87**, 90, **92**, **95**, **97**	167-169, 171, 182-184, 186	17
7	2013	-	**84**, **85**, 86, **89**, **95**, 96, 99, 105, 106, 109	-	10
8	2013	**71**, 74, 77, **78**, 79, 82, **83**	**84**, **85**, 88, **89**, 90, 93, 94, **95**, 98-100, 103, 104	169, 170, 174, 175	24
9	2013	63, 66, 80, **83**	**84**, **85**, **87**, **89**, 90, **92**, 94, **95**, **97**, 99, 100, 102, 104	167, 169-172, 174-177, 179-182, 184-187, 189, **190**, **191**	37

The peptide epitopes that showed ≥ 50% of ADCC antibody responses of all tested sera against each pool are shown in bold.

Table 3. Peptide mapping of high ADCC antibody sera against Gag peptide pools G1, G2, and G4.

Serum		Single peptide number of Gag pool responses to ADCC antibody			Total peptide responses
No.	Obtained year	Gag pool 1 (G1)	Gag pool 2 (G2)	Gag pool 4 (G4)	
4	2007	3778	-	3797-8, 3803-4, 3806	6
14	2007	-	**3789**, **3790**	3798-9, 3804-5	6
10	2008	-	-	3798-9, 3801, **3802**, 3804-5	6
15	2009	-	3784, **3789**, **3790**, 3742	**3800**, 3801	6
11	2011	3775	3784, **3790**	**3800**, **3802**	5
15	2011	-	3784, **3790**	-	2
16	2011	**3776**, **3779**	**3789**, **3790**, 3742	-	5
4	2012	3778, **3779**, 3780	3788, **3789**, **3790**	**3800**, **3802**, 3803	9
5	2012	-	3788, **3789**	-	2
7	2012	**3776**, **3779**	3786, **3789**	**3800**, 3803	6
9	2012	**3776**, **3779**	3784, **3789**, **3790**, 3742	-	6
12	2012	**3776**, **3779**	3784, 3786-7, **3789**, **3790**, 3742	3797, **3800**, 3806	11
17	2012	3775, **3776**, 3777-8, **3779**, 3780	3782-7, 3742	3803	14
5	2013	-	-	**3800**, **3802**	2
6	2013	-	-	3797, 3799, **3800**, **3802**, 3806	5

The peptide epitopes that showed ≥ 50% of ADCC antibody responses of all tested sera against each pool are shown in bold.

Table 3. (Continued) Peptide mapping of high ADCC antibody sera against Gag peptide pools G1, G2, and G4.

Serum		Single peptide number of Gag pool responses to ADCC antibody			Total peptide responses
No.	Obtained year	Gag pool 1 (G1)	Gag pool 2 (G2)	Gag pool 4 (G4)	
7	2013	-	-	**3800**	1
8	2013	3772-5, **3776**, 3777, 3781	3782-8, **3789**, **3790**, 3742	**3802**	18
9	2013	3775, **3776**, 3777-8, **3779**, 3780	-	3801, **3802**	8

The peptide epitopes that showed ≥ 50% of ADCC antibody responses of all tested sera against each pool are shown in bold.

Table 4. Sequences of potential ADCC antibody epitopes of HIV-1 CRF01_AE Env TH023/CM240

Peptide Pool - No.	Potential ADCC Ab epitopes of HIV-1 CRF01_AE gp160	Amino acid #	Number of sera respond to ADCC epitopes of group			Total sera responses (%)	Glycoprotein location
			High responses (%)	Medium responses (%)	Low responses (%)		
3 - 71	TNNAK TIIVH LNKSV	280-294	8/20 (40)	7/25 (28)	3/20 (15)	18/65 (27.69)	gp120 (C2)
3 - 78	SINIG PGQVF YRTGD	308-322	9/20 (45)	18/25 (72)	11/20 (55)	38/65 (58.46)	gp120 (V3)
3 - 83	RKAYC EINGT KWNEV	328-342	9/20 (45)	6/25 (24)	2/20 (10)	17/65 (26.15)	gp120 (V3-C3)*
4 - 84	CEING TKWNE VLKKV	332-346	7/20 (35)	6/25 (24)	9/20 (45)	22/65 (33.85)	gp120 (C3)
4 - 85	GTKWN EVLKK VTKKL	336-350	7/20 (35)	11/25 (44)	10/20 (50)	28/65 (43.08)	gp120 (C3)
4 - 87	KKVTK KLKEH FNNKT	344-358	8/20 (40)	7/25 (28)	6/20 (30)	21/65 (32.31)	gp120 (C3)
4 - 89	EHFNN KTIIF QPPSG	352-366	6/20 (30)	7/25 (28)	11/20 (55)	23/65 (35.39)	gp120 (C3)
4 - 92	PSGGD LEITM HHFNC	364-378	6/20 (30)	11/25 (44)	15/20 (75)	32/65 (49.23)	gp120 (C3)
4 - 95	FNCRG EFFYC NTTRL	376-390	7/20 (35)	8/25 (32)	2/20 (10)	17/65 (26.15)	gp120 (C3-V4)**
4 - 97	YCNTT RLFNN TCMEN	384-398	7/20 (35)	0/25 (0)	0/20 (0)	7/65 (10.77)	gp120 (V4)
7 - 190	LSFQT PSHHQ REPDR	708-722	8/20 (40)	5/25 (20)	1/20 (5)	14/65 (21.54)	gp41 (CT)
7 - 191	TPSHH QREPD RPEGI	712-726	8/20 (40)	5/25 (20)	5/20 (25)	18/65 (27.69)	gp41 (CT)

C = constant region; V = variable region; CT = cytoplasmic tail
*V3 (aa 328-331) - C3 (aa 332-342); **C3 (aa 376-384) - V4 (aa 385-390)

Table 5. Sequences of potential ADCC antibody epitopes of HIV-1 CRF01_AE Gag

Peptide Pool - No.	Potential ADCC Ab epitopes of HIV-1 CRF01_AE Gag	Amino acid #	Number of sera respond to ADCC epitopes of group			Total sera responses (%)	Protein location
			High responses (%)	Medium responses (%)	Low responses (%)		
1 - 3776	LERFA LNPSL LETTE GCQQI	41-60	7/20 (35)	6/25 (24)	2/20 (10)	15/65 (23.08)	p17
1 - 3779	GTEEL RSLYN TVATL YCVHQ	71-90	7/20 (35)	8/25 (32)	7/20 (35)	22/65 (33.85)	p17
2 - 3789	MFSAL SEGAT PQDLN MMLNI	171-190	9/20 (45)	3/25 (12)	6/20 (30)	18/65 (27.69)	p24
2 - 3790	PQDLN MMLNI VGGHQ AAMQM	181-200	9/20 (45)	5/25 (20)	7/20 (35)	21/65 (32.31)	p24
4 - 3800	CKSIL RALGA GATLE EMMTA	330-350	6/20 (30)	8/25 (32)	9/20 (45)	23/65 (35.39)	p24
4 - 3802	CQGVG GPGHK ARVLA EAMSQV	350-370	6/20 (30)	13/25 (52)	8/20 (40)	27/65 (41.54)	p24-p2*

p17 = MA (matrix); p24 = CA (capsid)
*p24 (aa 350-363) - p2 (aa 364-370)

were identified. For the parent G1 peptide pool, 7/10 (70.00%) sera showed ADCC activity against peptides 3776 and 3779. For the parent G2 and G4 pools, 9/12 (75.00%) and 7/14 (50.00%) of the sera showed ADCC activity against peptides (3789, 3790) and (3800, 3802), respectively.

The responses of medium- and low-ADCC antibody sera against the common ADCC antibody epitopes of HIV-1 CRF01_AE Env and Gag that were identified in high ADCC antibody sera

Twenty five medium- and 20 low-ADCC antibody sera were examined for their responses to the 12 and 6 potential common ADCC antibody epitopes of HIV-1 Env and Gag, respectively. For Env protein, the range of 5/25 (20%) - 18/25 (72%) and 1/20 (5%) - 15/20 (75%) of medium- and low-ADCC antibody sera, respectively demonstrated the responses to 11 common ADCC epitopes (peptides 71, 78, 83, 84, 85, 87, 89, 92, 95, 190, 191); i.e., except peptide 97 (**Table 4**). The overall responses of 65 tested sera against these peptides were > 20%, i.e., 21.54% - 58.46%. These 11 common epitopes located within the C2, V3, C3 and V4 of gp120 and the cytoplasmic tail (CT) of gp41 region.

For Gag protein, the range of 3/25 (12%) - 13/25 (52%) and 2/20 (10%) - 9/20 (45%) of medium- and low-ADCC antibody sera, respectively, demonstrated the responses to 6 common

ADCC epitopes (peptides 3776, 3779, 3789, 3790, 3800, 3802) (**Table 5**). The overall responses of 65 tested sera against these peptides were > 20%, i.e., 23.08% - 41.54%. These 6 common epitopes are localized within the p17, p24 and p2 regions of Gag protein.

Discussion

In this study, sixty five sera of young Thai men who enrolled to the conscripts were confirmed as sera from early HIV-1 CRF01_AE infected individuals using the Aware™ BED™ EIA HIV-1 Incidence Test (data not shown). Estimation of the mean duration of recent infection (MDRI) was 198.4 days for BED.[13] All sera were screened for high-, medium- and low-ADCC antibody activity against gp120 pulsed target cells utilizing an EGFP-CEM-NK^r flow cytometric assay. The gp120 was used instead of gp160 because only HIV-1 CRF01_AE gp120 is commercially available. Our assay measures the number of target cells that are killed.[10]

Identification of potential ADCC antibody epitopes using a matrix format led to the finding that the spectrum of peptide specific reactivity varied for each serum tested. All tested sera showed reactivity against a total of 1-37, and 1-18 peptides for Env and Gag proteins, respectively (**Tables 2, 3**). Studies on Env protein further showed that three epitopes (peptides 71, 78, 83) of pool E3, seven epitopes (peptides 84, 85, 87, 89, 92, 95, 97) of pool E4 and two epitopes (peptides 190, 191) of pool E7 were identified as being targets of ADCC antibodies from at least 50.00% of the tested sera (**Table 2**). Interestingly, all these potential epitopes except peptide 97 could be able to gain the responses from sera of medium- and low-ADCC activities for at least 20% and 5%, respectively which made the overall total responses to the range of 21.54-58.46% (**Table 4**). The aa sequences of the 12 potential ADCC epitopes of HIV-1 CRF01_AE gp160 are also shown in **Table 4**. The common peptides 71, 78, and 83 are localized within the C2, V3, and V3-C3 regions of gp120, respectively while the common peptides (84, 85, 87, 89, 92), 95 and 97 are localized within the C3, C3-V4 and V4 of gp120, respectively (**Figure 1**). In addition, the common peptides 190 and 191 are in the cytoplasmic tail region of gp41. Kijak et al. studied the molecular evolution of the HIV-1 epidemic among volunteers of RV144 vaccine trial from 2003 to 2009.[14] They found that CRF01_AE strains were 31% more diverse than the ones from the 1990s Thai epidemic. Thus, HIV-1 CRF01_AE strains that infected individuals in our study should also be diverse since the sera were obtained during 2001-2013. Interestingly, sera from these individuals could be able to demonstrate ADCC antibody responses to these common potent epitopes. This indicates that these ADCC epitopes were quite conserved and immunodominant.

Moreover, peptides 78, 85 and 92 showed the responses to ADCC activity at a larger number of sera of medium- and low-ADCC response groups than those of a high-ADCC response group, i.e., (72%, 55% vs 45%), (44%, 50% vs 35%), (44%, 75% vs 30%), respectively. This revealed the importance of these peptide sequences as the prominent ADCC epitopes for induction of ADCC responses against Env peptides of early HIV-1 infected individuals, i.e., 58.46%, 43.08% and 49.23%, respectively, of total 65 tested sera (**Table 4**).

Kulkarni et al. reported that 5 out of 10 Indian long-term non-progressors (LTNPs) demonstrated ADCC activity against the target epitopes in Env-C V3 region (aa 288-330: LNESVEI VCTRPNNNTRKSIRIGPGQTGDIIGDIRQAHC).[15] They proposed the potential role of this anti-V3 ADCC response in viral control which may contribute to the slower disease progressors. Their epitope sequences included our full epitope peptide 78 (aa 308-322: SINIGPGQVFYRTGD) and partial of epitope peptide 71 (aa 290-294:…LNKSV). The V3 region is one of the most immunogenic epitopes in the envelope which the V3 crown composed of the highly conserved aa I307, I309 and F317 that can induce specific antibody in > 90% of HIV infected individuals.[16,17] V3-specific Abs from RV144 vaccinees were shown to capture infectious virions.[18] Moreover,

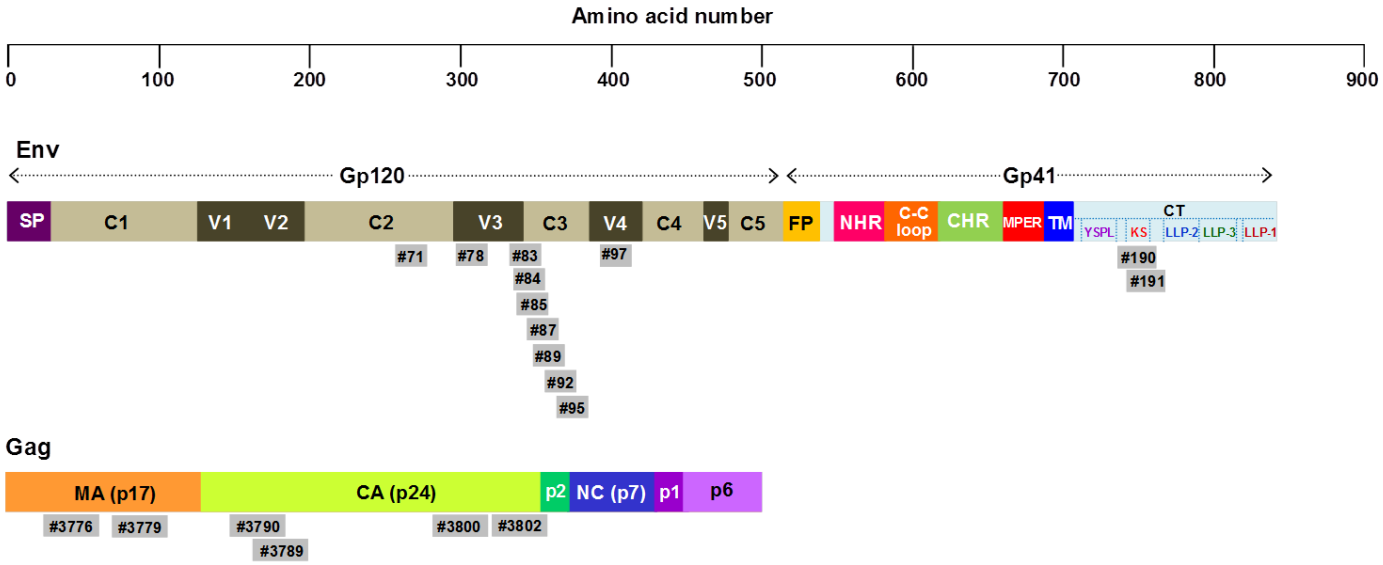

Figure 1. Epitope distribution across the Env gp160 and Gag of HIV-1. ADCC antibody epitopes of HIV-1 CRF01_AE identified in this study are shown. The epitope sizes are not drawn to scale and the amino acid locations of the epitopes are aligned as close as possible with the true alignment. C = constant region; V = variable region; CT = cytoplasmic tail; KS = Kennedy sequence; MA = matrix; CA = capsid

Montefiori et al. demonstrated the high Nt antibody activity against V3 peptide of HIV-1 CRF01_AE TH023 (aa 302-326: SNNTRTSINIGPGQVFYRTGDIIGD) from volunteer sera of RV144 phase III trial.[19] The initial ADCC antibody response to the V3 epitopes in our study may occur against the founder virus in most of the early HIV-1 infected individuals. However, the later consequence may depend on the efficiency of each individual immune response. If one can control the virus as in LTNP, the ADCC antibody against these epitopes should be detectable for a longer time period. On the other hand, the virus may mutate the V3 sequence to evade the immune responses[20] and later ADCC antibody may be further adapted to recognize the coming mutated V3 epitopes. Thus, the ADCC antibody at the non-early phase would not be able to respond to these original V3 epitopes as we previously reported on ADCC epitopes of HIV-1 in asymptomatic infected individuals.[12] Our finding of peptide 85 (aa 336-350: GTKWNEVLKKVTKKL) as dominant ADCC epitope was also correspondent to the data of Kulkarni et al.[15] They demonstrated the recognition of novel antigenic ADCC epitope in sera of LTNPs at the C3 region (aa 331-360: CNISEEKWNKTLQRVSEKLKEHFPNKTIKF).

The CD4-bound conformation of Env was recently shown to be a major target of ADCC-mediating antibodies present in the sera of HIV-1-infected individuals.[21] Prévost et al. reported the influence of the envelope gp120 Phe 43 cavity on HIV-1 sensitivity to ADCC responses.[22] While residue S375 in the gp120 Phe 43 cavity is well conserved in the majority of group M HIV-1 isolates, CRF01_AE strains have a naturally occurring histidine at this position (H375). They demonstrated that the H375 increases Env propensity to sample the CD4-bound conformation, thus increasing susceptibility of HIV-1 infected cells to ADCC. They raise the intriguing possibility that the presence of His 375 in the circulating CRF01_AE strain in Thailand might have contributed to the efficacy of the trial by spontaneously exposing epitopes recognized by ADCC -mediating antibodies elicited by the RV144 vaccine regimen. Our finding on peptide 92 (aa 364-378: PSGGDLEITMHHF NC) with His 375 emphasized the immunodominant property of this peptide as ADCC epitope since 49.23% of tested sera showed the ADCC responses.

Tomaras et al. reported that the first anti-HIV-1 antibody was the antibody against gp41 and appeared 13 days after the appearance of plasma virus.[23] In our study, the common peptides 190 (aa 708-722: LSFQTPSHHQREPDR) and 191 (aa 712-726: TPSHHQREPDRPEGI) which are located in the Kennedy sequence (KS) of cytoplasmic tail (CT) region of gp41 showed the ability to induce ADCC antibody with the total responses of 21.54% and 27.69%, respectively, of 65 tested sera (**Table 4**). This confirmed the study of Dimmock et al. on the alternative topological model of CT sequence: external localization.[24] Moreover, anti-KE (Kennedy epitope) serum that could specifically neutralize HIV in vitro implied the localization of the KE on the outer surface of the virion.[25] Abacioglu et al. revealed that the KS epitope PDRPEG was a conformational epitope which could induce non-Nt antibody (Chessie 8) binding to HIV-infected cells.[26] This indicated the property of ADCC antibody inducible of our peptides as we demonstrated in our study.

Identification of ADCC antibodies targeting conserved non-Env proteins such as Gag may reveal more potent ADCC activities. The appearance of HIV-core antigens on the surface of infected cells has been shown for p17 and p24.[27,28] Moreover, the monoclonal antibodies (mAbs) to p24-core protein of HIV-1 have been demonstrated to mediate ADCC activity to destroy infected cells.[29] Our studies on Gag protein further demonstrated that two epitopes of each peptide pools G1 (peptides 3776, 3779), G2 (peptide 3789, 3790) and G4 (peptides 3800, 3802) were identified as being targets of ADCC antibodies from at least 50.00% of the sera tested (**Table 3**). Interestingly, all these 6 epitopes could be able to gain the responses from sera of medium- and low-ADCC activities for at least 12% and 10%, respectively which made the overall responses to the range of 23.08-41.54% (**Table 5**). The aa sequences of the 6 potential ADCC epitopes of HIV-1 CRF01_ AE Gag are also shown in **Table 5**. The common peptides 3776 and 3779 are localized within the p17 (MA, matrix) region while the common peptides 3789, 3790 and 3800, 3802 are localized within the p24 (CA, capsid) region (**Figure 1**). Our finding of peptide 3789 (aa 171-190: MFSALSEGATPQD LNMMLNI) was correspondent to that of Grunow et al.[29] They showed the recognition of p24-ADCC epitope (aa 178-187: GATPQDLNIM) expressed on the HIV-infected T cells by mAbs. Interestingly, peptides 3779, 3800 and 3802 demonstrated the responses to ADCC activity at an almost equal/higher number of sera of medium- and low-ADCC response groups than those of a high-ADCC response group, i.e., (32%, 35% vs 35%), (32%, 45% vs 30%) and (52%, 40% vs 30%), respectively. This revealed the important of these peptide sequences as the dominant ADCC epitopes for induction of ADCC responses against Gag peptides of early HIV-1 infected individuals, i.e., 33.85%, 35.39% and 41.54%, respectively, of total 65 tested sera. Genetic data support a major role for the cytoplasmic tail of gp41 and the matrix domain of Gag in Env glycoprotein incorporation.[30] Both Gag and Env display properties of being associated with lipid rafts at the plasma membrane. Gag-Env interactions affect their co-recruitment to the virological synapse and subsequent early events in the next round of viral replication. The ADCC antibody against epitopes on matrix domain of Gag demonstrated in our study may have an effect on the new coming viral progeny at the Env incorporation process.

In conclusion, although the degree of ADCC antibody responses to the gp120 protein varied from high to low, there were certain potent consensus Env and Gag peptide epitopes that could induce the ADCC antibody responses in a majority of early infected individuals as shown in this study. This information should be useful for further design of the antibody epitope based vaccine. The pre-existing potent antibody induced by the vaccine should be able to limit the transmitted/founder viruses, delay acquisition, control viral replication and prevent the establishment of infection. Since cell-to-cell transmission appears to be more efficient than that of free virion for HIV-1 infection,[31] the ADCC responses would be a very important mechanism to eliminate the small number of virus-infected cells from an infected partner before the establishment of infection. Although, Nt antibodies have

no effect on cell-to-cell transmission of HIV-1,[32] their responses would be very crucial for eliminating free virions that may enter simultaneously with the viral infected cells during viral transmission. This warrants further study whether these ADCC epitopes also act as Nt antibody epitopes. Our studies are limited by the use of peptide/protein pulsed target cells instead of HIV-1 infected cells. The 3-dimentional structure of Env or Gag on the target cell surface may be not the same as those on the infected cells.

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Author's contributions: SA, SS, ST and NH were contributors for performing experiments including collection of data, analysis; TC and PE oversaw the collection of data, analysis and interpretation; WK is the corresponding author and is the main contributor to the design and direction of the study including writing the manuscript.

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ORIGINAL ARTICLE

Lymphocyte subsets and natural killer cell cytotoxicity in intravenous drug users with HIV-1 infection among Thai population

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Abstract

Background: Intravenous drug users (IVDUs) are among the high-risk groups who are most vulnerable to HIV infection. Several illicit drugs alter host immune function with increased incidence of infections including that of HIV. Many studies of the immune response of NK cells in HIV-1 seronegative IVDUs and HIV-1 seropositive IVDUs have been published from the Western countries and yet no data is available from Thailand.

Objective: To determine natural killer cell cytotoxicity and lymphocyte subsets in Thai HIV-1 infected intravenous drug users.

Methods: The NK cell cytotoxic function was determined using our well-established EGFP-K562 flow cytometric assay in 30 IVDUs with HIV-1 infection (IVH) comparing with those from the same number of non-infected IVDUs (IVX), HIV-1 seropositive individuals (HIV-1+ve) and healthy controls. The percentage and the absolute number of NK cells, helper $CD4^+$ T cells and cytotoxic $CD8^+$ T cells were also investigated.

Results: Among the study groups, IVH showed not only the lowest percentage of lytic activity by NK cells, but also a decline in the percentage and absolute count of NK cells. A decline in helper $CD4^+$ T cells and an increase of cytotoxic $CD8^+$ T cells of IVH group when compared to those of other 3 groups were also demonstrated.

Conclusions: The failure of innate immune NK cell function and their number in IVH may support the involvement of additional components of the immune system in the control of HIV-1 disease.

Key words: NK cells; NK cell cytotoxicity; $CD4^+$ T cells; $CD8^+$ T cells; HIV-1; intravenous drug users

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Introduction

Human immunodeficiency virus (HIV)/AIDS is still a major global health problem with an estimated 35.3 million people are living with HIV.[1] In Thailand, more than 1 million of HIV-1 infected people were reported in 2015.[2] Of the estimated 440,000 people living with HIV, the relatively high levels of prevalence were in men who have sex with men, transgender people, male/female sex workers and their partners, migrant workers and intravenous drug users (IVDUs). Interestingly, the HIV-1 prevalence among the Thai IVDUs has the highest HIV-1 prevalence (about 30-50%) comparing to other risk groups of HIV-1 infection[3] with predominantly a CRF01_AE subtype.[4-6] It has been known that illicit drugs alter immune unction and decrease host resistance to microbes with increased incidence of infections or immune disorders in humans, including infection with HIV and disease progression to AIDS.[7,8]

Human natural killer (NK) cells are large granular lymphocytes of the innate immune system.[9] NK cells are recognized as a subset of cytotoxic innate lymphoid cells which play a role in the killing of tumor and virus-infected cells and participate in shaping the adaptive immunity by secretion of cytokines.[10] NK cells are cytotoxic with small granules in their cytoplasm contain proteins such as perforin and proteases known as granzymes, these proteins are released when they encounter with an infected cell by inducing either apoptosis or osmotic cell lysis.[11,12] NK cells can also recognize and lyse target cells by antibody dependent cell-mediated cytotoxicity (ADCC). The increased risk for the disease progression in HIV-infected individuals are associated not only with the low number of the NK cells but also with the defect in their cytotoxic activity[11] and also with decline of helper $CD4^+$ T cells and the accumulation of cytotoxic $CD8^+$ T cells.[13,14]

Although there are many studies of the innate immune response of NK cells in HIV-1 seronegative IVDUs and HIV-1 seropositive IVDUs,[15-18] the study of NK cells due to the HIV-1 infection and/or intravenous drug abuse in Thais has not been conducted. In this study, we used our well-established enhanced green fluorescent protein (EGFP)-K562 flow cytometric method[19] for measuring the NK cell function in IVDUs with HIV-1 infection (IVH). Results were also compared with those from HIV-1 infected individuals (HIV-1+ve) and non-infected IVDUs (IVX) as well as healthy individuals.

Methods

Study population and blood samples

Thirty HIV-1 uninfected IVDUs, 22 males and 8 females, and 30 HIV-1 infected IVDUs, 24 males and 6 females, at Public Health Center 3 (Bang Sue), Health Department, Bangkok Metropolitan Administration were enrolled in this study with written informed consents. All volunteers were documented to have treatment for drug addiction. All 30 HIV-1 infected IVDUs had previously tested once for HIV-1 antigen in saliva at this center. Fresh venipuncture Acid citrate dextrose solution B (ACD, Becton Dickinson Biosciences {BDB}, CA, USA) blood samples and Tripotassiumethylenediamine tetra-acetate (K_3EDTA, BDB) blood samples from each subject were collected. Complete blood counts (CBC) of each blood sample was performed at the laboratory of Department of Clinical Pathology, Faculty of Medicine Siriraj Hospital by using automate hematological analyzer Sysmex XE-5000 (Sysmex Corporation, Kobe, Japan).

Leuko-pak and K_3EDTA blood from 30 asymptomatic HIV-1 infected blood donors, 22 males and 8 females, were kindly provided by the Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital and Thai Red Cross. Thirty healthy HIV-1 seronegative blood subjects, 23 males and 7 females were also recruited from the blood bank donors. All samples obtained had no donor identifiers. There were no differences in the base line characteristics of all 4 study groups, i.e., age, sex ratio.

This study was approved by Ethics Committee of the Bangkok Metropolitan Administration for IVDUs subjects (No. 147-2009) and Institutional Review Board, Faculty of Medicine Siriraj Hospital (COA: Si031/2007) for all samples.

Immunophenotyping staining of peripheral blood samples

Percentages and absolute cell numbers of NK cells ($CD3^-CD16^+CD56^+$), helper $CD4^+$T cells ($CD3^+CD4^+$) and cytotoxic $CD8^+$T cells ($CD3^+CD8^+$) were determined by standard TriTESTmethod[20,21] using BD Tritest™ monoclonal antibody (mAb) reagents. Briefly, ten µl of TriTEST 3-color mAb reagents were mixed with 50 µl of EDTA-anticoagulated whole blood and incubated for 20 min at room temperature in the dark before adding 450 µl of FACS Lysing Solution (BDB). After an incubation time of 15 min, the cells were washed at 1,400 rpm for 5 min. The cell pellet was resuspended in 300 µl of 1% paraformaldehyde and kept at 2-8°C until analysis by FACSCalibur flow cytometer (BDB). Percentages of NK cells, helper $CD4^+$ T cells and cytotoxic $CD8^+$ T cells were obtained by using CellQuest™ Analysis software (BDB). The absolute cell numbers of these lymphocyte subsets were determined by multiplying the percentage of each subset with the absolute lymphocyte counts from the CBC.

Preparation of effector cells

Peripheral blood mononuclear cells (PBMCs) from each ACD blood sample were separated by standard ficoll-hypaque density gradient centrifugation, washed three times with 0.2 M phosphate buffered saline (PBS) and resuspended in freezing medium containing 60% RPMI 1640 (Gibco Laboratories, Grand Island, NY, USA), 30% fetal bovine serum (FBS, Gibco Laboratories) and 10% dimethyl sulfoxide (Sigma-Aldrich Corp, MO, USA). Aliquots of PBMCs at a density of $5x10^6$ cells/ml were cryopreserved in liquid nitrogen until use. One day prior to the experiment, PBMCs were thawed and added to 100 mm polystyrene tissue culture plates (Griener Bio-One GmbH, Frickenhausen, Germany) and incubated overnight at 37°C with 5% CO_2 to deplete adherent monocytes. The non-adherent lymphocytes were collected and resuspended at a density of $1x10^6$ for use as effector cells in the NK cell cytotoxicity assay.

Preparation of target cells

The EGFP-K562 cell line was prepared as previously described[19] and used as targets for measuring NK cell cytotoxicity by flow cytometry. This cell line was cultured in growth medium containing RPMI 1640 supplemented with 2 mM L-glutamine (Gibco Laboratories), 100 µg/ml streptomycin (Gibco Laboratories), 100 IU/ml penicillin (Gibco Laboratories), and 10% heat inactivated FBS (Gibco Laboratories) and 400 µg/ml of neomycin analogue G-418 (Roche Diagnostics, IN, USA). Prior to the experiment, the target cells were resuspended in growth medium at a density of 1×10^5 cells/ml.

NK cytotoxicity flow cytometry assay

The EGFP-K562 flow cytometric assay for measuring NK cytotoxicity was based on a method previously described.[19] Briefly, four hundred microliters of PBMCs as effector cells at a density of 1×10^6 cells/ml were added into individual round bottom 12 × 75 mm polystyrene (Falcon) tubes (BDB) with 2-fold serial diluted in 10% RPMI 1640 growth medium. Fifty microliters of EGFP-K562 target cells as a density of 1×10^5 cells/ml were added into each tube to yield the effectors per

target cells (E/T) ratios of 80:1 and 40:1. All sample tubes were mixed and incubated at 37°C with 5% CO_2 for 4 hours. After incubation, propidium iodide (PI) (Sigma-Aldrich Corp) solution at a concentration of 100 µg/ml was added into the tubes to stain compromised or dead cells and then the tubes were incubated at room temperature for 10 minutes. The cytotoxic activity of NK cells to lyse target cells was measured by using a FACSCalibur flow cytometer (BDB). For measuring the percent lysis of NK cytotoxicity, exactly 2×10^4 events per test sample were examined and no gating was used for acquisition. The threshold for side scatter (SSC) was set on the linear scale to discriminate the debris and dead cells. Two parameters of the forward and side scatter (FSC/SSC) dot plots signals were analyzed by using CELLQuest™ software (BDB) (**Figure 1A**). The EGFP versus PI data were obtained to evaluate live and dead target cells using log scale of green fluorescent (FL1) or EGFP in the X axis and the log scale of red fluorescent (FL2) or PI in the Y axis (**Figure 1B**). The strong green fluorescent at the lower right (LR) quadrant were identified as the live target cells whereas the red and green fluorescent on the upper right (UR) quadrant were identified as compromised or dead target cells. The red fluorescence on the upper left (UL) quadrant were defined as the autodamaged or potentially lysed effector cells or target cells with decreased EGFP whereas the lower left (LL) were considered to be the living effecter cells or living target cells with weak green fluorescence. Effector cells alone and target cells alone stained with PI were used as controls. The percentage of target cell lysis was calculated by using the following formula: % Lysis = (UR/UR + LR) × 100.

Statistical analysis

Statistical analysis in this study was conducted on SPSS software version 11.5 (SPSS Inc., NY, USA). Statistical significance within group was analyzed by Shapiro-Wilk test and statistical significance between groups was analyzed with one -way ANOVA test. The significant level of each test between groups was adjusted by multiple testing using Bonferroni and Tamhane correction for homogeneity and heterogeneity of variance, respectively. The significant threshold of four comparisons of each test was set at p value < 0.05.

Results

Characteristics of study populations

Characteristics of Thai HIV-1 seronegative individuals (Healthy), HIV-1 seronegative IVDUs (IVX), HIV-1 seropositive individuals (HIV-1+ve) and HIV-1 seropositive IVDUs (IVH) in this study were shown in **Table 1**. Healthy group did not have any vaccination at least one year prior to this study. In IVX group, there was only one participant with history of Hepatitis B virus infection. All subjects in IVH group had

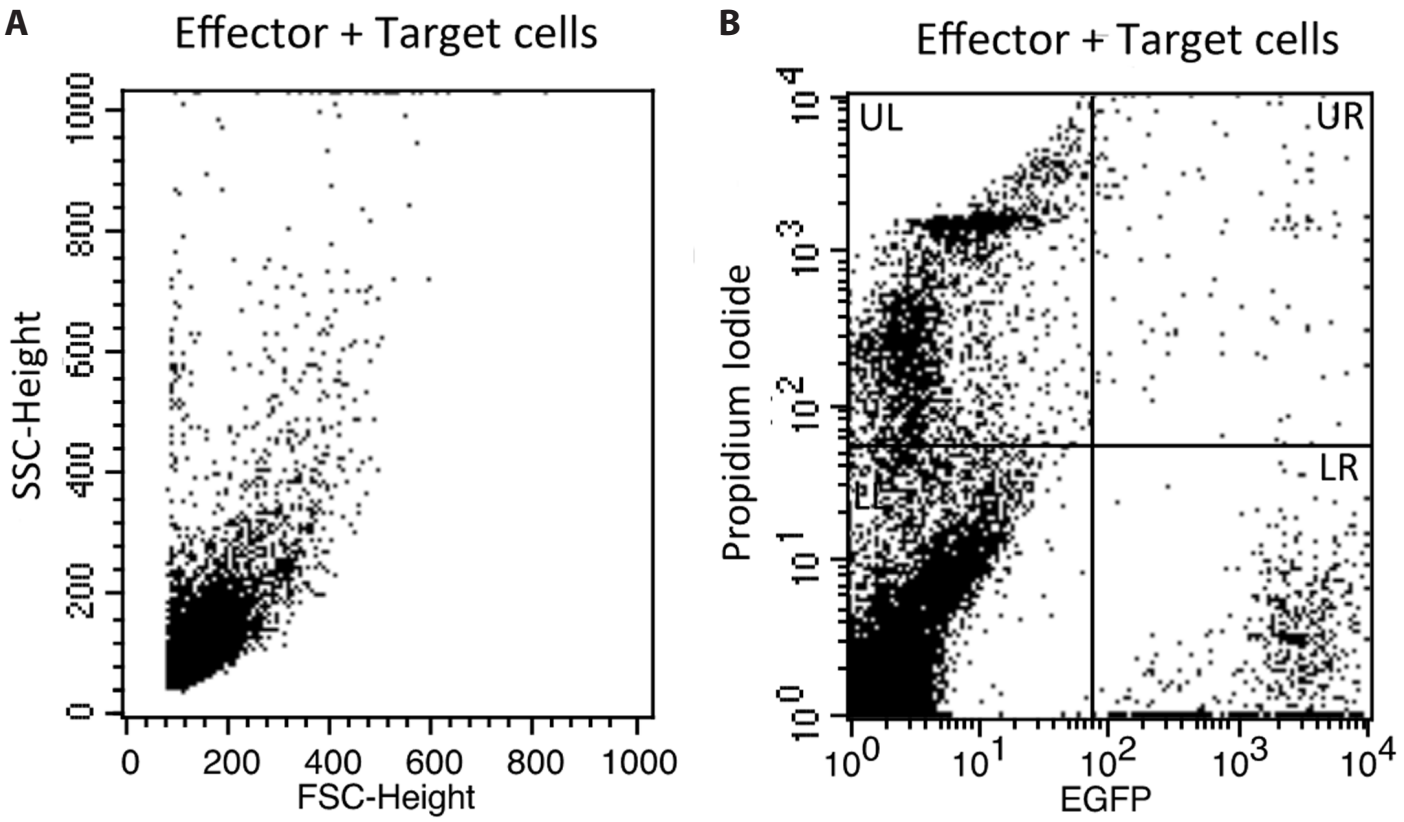

Figure 1. Representative flow cytometric two-parameter dot-plots on NK cytotoxicity test showing Forward Scatter (FSC) vs. Side Scatter (SSC) of NK (effector) cells and EGFP-K562 (target) cells A) and logarithmic EGFP vs. propidium iodide expression of live and dead effector vs. target cells (B). Cells at the lower right (LR) quadrant are lived target cells whereas the red and green fluorescent on the upper right (UR) quadrant are dead target cells. Cells at the upper left (UL) quadrant are defined as the autodamaged or potentially lysed effector cells or target cells with decreased EGFP whereas the lower left (LL) quadrant represents the living effecter cells or living target cells with weak green fluorescence.

Table 1. Demographics and characteristics of the study populations.

Group	Subjects	Age (yrs) Mean ± SD	Gender		Injecting drug (%)			Inhalation drug (%)	
			Male	Female	Heroin	Methamphetamine	Domicum	Methamphetamine	Cannabis
Healthy	30	30 ± 6.7	23	7	-	-	-	-	-
IVX	30	39 ± 6.9	22	8	66.6	52.4	28.6	68.8	56.3
HIV-1+ve	30[a]	31 ± 9.1	22	8	-	-	-	-	-
IVH	30[b]	39 ± 8.1	24	6	37.5	58.3	54.2	83.3	61.1

IVX = HIV-1 seronegative IVDUs; IVH = HIV-1 seropositive IVDUs
[a] unknown history of antiretroviral drug treatment; [b] all subjects received tenofovir as antiretroviral drugs.

received tenofovir as antiretroviral drugs. All IVDUs subjects had history for multiple intravenous and inhalation drug behaviors. Their drugs used within the 3-month period prior to blood collection were mainly heroin, methamphetamine, domicum and cannabis for both IVX and IVH groups. Within the IVX group, the percentage of participants that used injecting drugs was 66% heroin, 52.4% methamphetamine and 28.6% domicum, and inhalation drugs was 68.8% methamphetamine and 56.3% cannabis (**Table 1**). For the IVH group, the percentage was 37.5% heroin, 58.3% methamphetamine and 54.2% domicum, and inhalation drugs was 83.3% methamphetamine and 61.1% cannabis (**Table 1**).

Percentages and absolute counts of NK cells, helper CD4⁺ T cells and cytotoxic CD8⁺ T cells in the study populations

Mean percentages and absolute counts of each lymphocyte subset among the study populations were compared and shown in **Table 2**. The mean percentages of helper CD4⁺ T cells of IVH group and HIV-1+ve group were significantly lower than those of IVX group and healthy group. No statistically significant difference among the two HIV-1 infected groups (IVH group vs. HIV-1+ve group) and among the two HIV-1 seronegative groups (IVX group vs. healthy group) were found. The mean absolute counts of helper CD4⁺ T cells from IVH group were significant decreased ($p \leq 0.001$) when compared with those from HIV-1+ve, IVX, and the healthy groups. Interestingly, the absolute counts of helper CD4⁺ T cells from IVX group were significantly higher than those of healthy and HIV-1+ve groups (p value ≤ 0.05) while those of healthy and HIV-1+ve groups were comparable.

For cytotoxic CD8⁺ T cells, the mean percentages in HIV-1 +ve group and IVH group were significantly higher ($p \leq 0.05$) than those of healthy group and IVX group. No statistically significant differences among the HIV-1+ve and IVH groups and among the IVX and healthy groups were seen (**Table 2**). Comparison of the absolute counts of cytotoxic CD8⁺ T cells among the study groups showed that HIV-1+ve group had a significant increase ($p \leq 0.05$) in the absolute counts when compared with those of IVH, IVX and the healthy groups. Moreover, no significant difference among the healthy, IVX and IVH groups were observed.

Among the study groups, the mean percentage of NK cells was significantly decreased ($p \leq 0.05$) in IVH group when compared to those from healthy group but did not show any significant difference to those of HIV-1+ve group and IVX group. No significant differences in mean percentages of NK cells were found among the HIV+ve, IVX and the healthy groups (**Table 2**). For the absolute counts of NK cells, the IVH group showed significant decrease ($p \leq 0.05$) in the absolute counts when compared with those of HIV+ve, IVX and the healthy groups. No significant differences among the healthy, IVX and HIV-1+ve groups were found.

Table 2. Comparison of percentages and absolute counts of helper T cells ($CD3^+CD4^+$), cytotoxic T cells ($CD3^+CD8^+$) and NK cells ($CD3^-CD16^+CD56^+$) in the 4 study groups.

Parameter	Mean ± SD				*p*-value
	Healthy gr.	IVX gr.	HIV-1 +ve gr.	IVH gr.	
Helper T cells					
%	33.25 ± 5.58	36.39 ± 8.78	22.39 ± 9.71*	19.99 ± 6.36*	≤ 0.05
cells/μl	784 ± 261	1104 ± 526	732 ± 581	358 ± 206**	≤ 0.001
Cytotoxic T cells					
%	30.61 ± 6.07	26.07 ± 8.16	49.38 ± 13.21*	51.09 ± 9.52*	≤ 0.05
cells/μl	657 ± 268	757 ± 390	1380 ± 874*	948 ± 406	≤ 0.05
NK cells					
%	18.99 ± 5.30	15.62 ± 7.74	14.55 ± 10.87	11.10 ± 6.47$	≤ 0.05
cells/μl	458 ± 189	451 ± 303	380 ± 320	188 ± 115**	≤ 0.05

* Statistical difference when compared to the Healthy and IVX groups.
** Statistical difference when compared to the Healthy, IVX and HIV+ve groups.
$ Statistical difference when compared to the Healthy group.

Table 3. Comparison of NK cytotoxicity function in the 4 study groups.

E/T ratio	Mean ± SD of % lysis of NK cytotoxicity in				*p*-value
	Healthy gr.	IVX gr.	HIV-1 +ve gr.	IVH gr.	
80:1	41.78 ± 7.99	52.02 ± 13.68	41.06 ± 11.23	33.77 ± 10.02*	≤ 0.05
40:1	30.91 ± 8.77	35.58 ± 15.40	29.64 ± 8.29	23.20 ± 9.71*	≤ 0.05

* Statistical difference when compared to the Healthy, IVX, and HIV+ve groups.

NK cell cytotoxicity in the study populations

Comparison of the mean percentages of EGFP-K562 target cell lysis at the E:T ratios of 80:1 and 40:1 by NK cells from the 4 study groups showed that the IVH group showed significantly decreased of NK cytotoxicity ($p \leq 0.05$) at the E:T ratio of 80:1 and 40:1 when compared to the other groups (**Table 3**). Interestingly, the mean lysis percentages of NK cytotoxicity in IVX group at both E/T ratios were significantly higher ($p \leq 0.05$) than those in HIV-1+ve, IVH and the healthy groups, particularly at E:T ratio of 80:1. Moreover, no difference of the mean percentages lysis activity between the healthy and the HIV-1+ve groups were shown.

Discussion

It is estimated that worldwide there are almost 13 million people who are IVDUs. Out of these nearly 2 million, or 15%, are also living with HIV which is typically far greater than it is among the rest of the adult population, with IVDUs bearing a 28 times higher prevalence.[22] In Thailand, there were an estimated 440,000 people living with HIV in Thailand in 2015 with an estimation of adult HIV prevalence of 1.1% out of Thailand's population of more than 60 million. Among 6,900 new HIV infections in 2015, unsafe injecting drug use is the second biggest transmission route which account for 12%.[23] During HIV-1 infection several immunological abnormalities have been found in IVDUs.[5,8] It has been known that the increased risk for the disease progression in HIV-infected individuals is associated with the decline of helper $CD4^+$ T cells and the accumulation of cytotoxic $CD8^+$ T cells with a more rapid helper $CD4^+$ T cell decline among HIV-1 infected IVDUs.[24] Biological factors such as effect of opioids, co-infection with other diseases such as TB or hepatitis C, even a difference in tropism with virulent strain of HIV transmitted among the IVDUs are all responsible for the faster decline in helper $CD4^+$ T cells.[25,26] A decline in both percentages and absolute counts of helper $CD4^+$ T cells with marked increases in percentages and absolute counts of cytotoxic $CD8^+$ T cells in our IVH group support the above findings. Although there was no history of how fast the decrease of helper $CD4^+$ T cells in our IVH group as only one blood sample at one time point was obtained, our IVH group showed more helper $CD4^+$ T cell decline than those in HIV+ve group. This may indicate the problems with an irregular adherence to antiretroviral drugs, loss of follow-up or lower access to health services in our IVH group. Unlike the decline helper T cells found in IVH group, our HIV+ve group showed higher helper $CD4^+$ T cell counts than the IVH group implying that there was a good suppressing HIV replication in these asymptomatic HIV-1 infected blood donors. Unfortunately, the history of antiretroviral drug treatment was not available for this group.

NK cells have been described for the innate mechanism as the first line defense against HIV-1 infection and may help mediating adaptive immune responses.[10-12] It has been shown that NK cell effect or function that mediated NK cell cytotoxicity are impaired during the course of HIV-1 disease[26] but enhanced during HIV-1 viremia.[27] Previous studies on the NK profiles from the cohort of HIV-1 exposed, uninfected IVDUs demonstrated the enhancement of NK activity, high NK activation as represented by $CD38^+$ cells, and the CD4 and CD8 cell-mediated resistance to HIV-1 infection.[18,28,29] However, there is limited information available regarding the effect of HIV-1 infection on the NK profile in Thai IVDUs. Our NK cytotoxicity study in IVDUs showed significantly decreased of lysis activity in IVH group when compared to those of healthy, HIV-1+ve and IVX groups. When defining the percentage and absolute count of NK cells, our study showed significant decrease of both the percentage and the absolute count of NK cells in IVH group when compared to those of healthy, HIV-1+ve and IVX groups. This data suggested the inverse effect to the NK cell numbers due to HIV-1 infection along with the use of injecting drugs. Our findings also support that the drug abuse has immune-modulating effects which could alter the progression of HIV infection.[30,31]

Of interest is that the NK functional activity in our IVX group was significantly increased when compared to the other 3 groups. The increased NK cell lysis activity in the IVX who are a high-risk group, could due to different mechanisms related either to immune responses and/or to genetic background contribute to the resistance to infection in the highly exposed but uninfected (EU) IVDUs. Our results support the contentions of enhanced innate immune cell function in EU individuals especially when compare with IVDUs who became HIV-1 infected.[28] There was no difference of the mean percentages NK celllys is activity between the healthy and the HIV-1+ve groups. This may be due to the asymptomatic stage of our HIV+ve individuals whose NK cell functions were still normal. When defining the percentage and absolute count of NK cells, our study demonstrated that not only there was no significant difference of the mean percentages of NK lysis activity among the HIV+ve, IVX and the healthy groups, but also there was no difference in percentages and the absolute counts of NK cells. Our findings are in line with de Souza et al. who reported that NK cell cytotoxicity and NK cell counts are not different between Thai HIV-1 seronegative and Thai HIV-1 seropositive subjects.[32]

In conclusion, our study shows that IVHs have alterations in the lymphocyte subsets evidenced by a decrease in helper

$CD4^+$ T cells and an increase in cytotoxic $CD8^+$ T cells along with a lower number of NK cells and their impaired cytotoxic function. The data suggest that the failure of innate immune NK cell function and their number with possible link to decrease in helper $CD4^+$ T cells and increase in cytotoxic $CD8^+$ T cells, may contribute to the ineffective immune surveillance and potentially predisposing them to progressive disease.

Acknowledgements

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Author's contributions: SJ and SA were contributors for performing experiments including collection of data, analysis; PB and RC were responsible on recruitment of IVDUs and blood collection. RS oversaw the collection of data, analysis and interpretation; WK and KP were the main contributors to the design and direction of the study including writing the manuscript. KP is the corresponding author.

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Abstract must be written in a structured format with the following headings: background; objective; methods; results; and conclusion. The major points of the article should be summarized in 150 (case reports) to 250 words (original research and review articles), in the order of their appearance in the manuscript. Abbreviations should be kept to an absolute minimum. References are not allowed in the abstract.

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