Dental Journal
Majalah Kedokteran Gigi
Volume 46 Number 2 June 2013

Research Report

New concept in allergy: Non-allergic rats becomes allergic after induced by *Porphyromonas gingivalis* lipopolysaccharide

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ABSTRACT

Background: *As a theory, seemingly it is impossible that allergic diseases, including asthma, are the result of exposure to a transmissible agent. The fact that nearly all children with asthma are allergic, but only a small proportion of allergic children have asthma, at least raises the possibility that other factors are involved. Interestingly, non-allergic children become allergic after their parents came from working in allergic people for several months. Recent research revealed that periodontal pathogens are also transmissible from mother and caregivers to infants.Therefore, it is logical that non-allergic children could become allergic after exposed to periodontopathic bacteria. However, the mechanism is still unclear.* ***Purpose:*** *The objective of this study is to verify a new concept that non-allergic rat may become allergic after exposed to Porphyromonas gingivalis lipopolysaccharide.* ***Methods:*** *Randomized control series design experimental study was conducted to 24 male Wistar rats, two experimental groups and one control group. One group was subjected to intrasulcular injection of $PgLPS_{1435/1450}$. Tissue examination were done for allergy biomarkers with peroxidase immunohistochemistry for leukotriene C4 (LTC4) and eosinophilic cationic protein (ECP) in bronchus tissue. Serum level examination of interleukin 4 (IL-4), and immunoglobulin E (IgE) was done with ELISA. Data were analyzes using ANOVA.* ***Results:*** *after four days, LTC4 and ECP expression increased significantly (p=0.001); even insignificant, IL-4 and IgE serum level also increased.* ***Conclusion:*** *PgLPS is able to stimulate immunocompetent cells which changed the host immune response of non-allergic rats. Therefore, it is possible that they become allergic.*

Key words: *Transmission, allergic, periodontopathic bacteria, lipopolysaccharide*

ABSTRAK

Latar belakang: *Menurut teori, penularan penyakit alergi termasuk asma merupakan hal yang mustahil. Fakta menunjukkn bahwa hampir semua anak penderita asma mempunyai alergi, tetapi tidak semua anak alergi menderita asma, sehingga mungkin ada faktor lain ya ng terlibat. Hal yang menarik adalah timbulnya gejala alergi pada anak non-alergi setelah orang tua mereka bekerja beberapa bulan pada orang yang alergi. Penelitian mutakhir juga menemukan bahwa bakteri periodontopatogen juga dapat ditularkan ke bayi dari ibu dan pengasuhnya. Sebagai akibatnya, sangat nasuk akal bila anak non-alergi menjadi alergi setelah terpajan bakteri periodontopatogen.* ***Tujuan:*** *Untuk verifikasi konsep baru, yaitu bahwa tikus non-alergi dapat menjadi alergi setelah terpajan lipopolisakarida.* ***Metode:*** *Pada 24 tikus Wistar jantan; dua kelompok perlakuan dan satu kontrol. Satu kelompok diberikan injeksi intrasulcular dengan $PgLPS_{1435/1450}$. Pemeriksaan jaringan dilakukan pada biomarker alergi menggunakan imunohistokimia peroxidase untuk leukotriene C4 (LTC4) dan eosinophilic cationic protein (ECP) dari jaringan bronkus. Pemeriksaan kadar serum pada interleukin 4 (IL-4), dan immunoglobulin E (IgE) menggunakan metode ELISA. Data dianalisis dengan ANOVA.* ***Hasil:*** *Setelah empat hari, LTC4 ekspresi ECP meningkat secara bermakna (p=0.001); walau tidak bermakna, kadar IL-4 and IgE serum juga meningkat.* ***Simpulan:*** *PgLPS dapat merangsang sel imunokompeten sehingga dapat merubah respons imun tikus non-alergi menjadi alergi.*

Kata kunci: *Penularan, alergi, bakteri periodontopatogen, lipopolisakarida*

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INTRODUCTION

Atopy or allergy is a personal and/or familial tendency to, usually in childhood or adolescence, become sensitized and produce immunoglobulin E (IgE) instead of IgM or IgG antibodies in response to ordinary exposure to low doses of allergens, usually proteins. However, IgE normally existed in non-allergic individuals eventhough it was the least amount among other immunoglobulins.[1] Atopy can be detected by specific serum IgE or skin-test reactivity to environmental allergens and it is often associated with asthma. In some populations, the prevalence of asthma associated with allergies has increased more than that of non-atopic asthma, whereas in others the prevalence of the two types of asthma has increased to a similar degree. Therefore, until today it is still not exactly known what factors cause asthma in a person with atopy or what factors cause atopy in a person with asthma.[1,2] Clinical studies of children and interventional studies of animals indeed suggest that the exposure to microbes through the gastrointestinal tract was able to develop immune function. In addition, the initial microbial exposure for children born by Caesarean section is delayed compared with those born by vaginal delivery; thus have more asthma risk.[2,3]

This phenomenon was called as the "hygiene hypothesis" introduced by David Strachan in 1989, it proposed that the increase in allergic diseases was caused by decrease o exposed infections during childhood.[1] Nevertheless, current cohort studies suggest that the risks of asthma are increased in children who suffer severe illness from a viral respiratory infection in infancy. Moreover, researches revealed that asthma appeared first in adults returning to villages after working in a European influenced city. Only thereafter did it appear in children; and another has found the rates of asthma to be nearly as high in adopted children of mothers with asthma as in natural children.[4] As the result, Yoo *et al.*[4] in 2007 proposed a new concept that asthma is a transmissible disease.

The possibility that oral bacteria infection may induce allergic asthma was confirmed by Wiyarni *et al*.[5] as well as Utomo and Harsono[6] studies. It revealed that dental plaque control therapy improves respiratory quality and that the asymptomatic asthmatic children had significant lower Gram-negative positive culture than uncontrolled asthma (wheezing and coughing)[5] as well as significant decrease of histamine serum level[6] in one week study. Evaluation of randomly selected subjects after two months reported that their asthmatic symptoms and food allergy also diminished.[7] Coincidentally, a study by Lee *et al*.[8] showed that the periodontopathic bacteria in dental plaque are also transmissible. It was revealed that if at least one of the parents harbored a periodontal pathogen, then the child will exhibit the same genotype of bacteria. Therefore, there is a possibility that allergic asthma is not merely inherited, but also transmissible to non-allergic individuals via periodontopatic bacteria.

Tanner *et al*.[9] found that various anaerobic species colonize the edentulous mouths of infants, and that maternal or caregivers saliva may act as a source of some Gram-negative anaerobes.[7] Several literatures had been suspected the involvement of periodontopathic bacteria in the pathogenesis of allergy such as Netea *et a.l*,[10] Kato *et al.,*[11] and Card *et al.,*[12] reported that allergic immune response could be modulated by Gram-negative periodontopathic bacteria, could be worsen or attenuate the symptoms, depended upon the time of exposure. However, they still used ovalbumin (OVA) injection for allergy sensitization while our study only used OVA inhalation

It was interesting that periodontopathic bacteria lipopolysaccharides (LPS) have a unique characteristics; in some instances the enhanced type 1 immune response which release interferon-γ, i.e. LPS from *Aggretibacter actinomycetemcomitans* and *Porphyromonas gingivalis* (high dose, ≥ 1.0 μg/mL); nevertheless low dose of *P. gingivalis* LPS (*Pg*LPS) (< 1.0 μg/mL) enhance the Th2 immune response which release interleukin-4 (IL-4) and IL-13.[10] Nevertheless, according to Kumada *et al.*,[14] synthetic LPS only stimulated TLR4, thus was a TLR4-ligand. Nevertheless, Darveau *et al*,[15] reported that $PgLPS_{1435/1450}$ a particular synthetic *Pg*LPS with major Lipid A mass ion 1435 and 1450 m/z ($PgLPS_{1435/1450}$) could act as an immunomodulator either to TLR2 or TLR4. Therefore, in this study, it was used for mimicking naïve *Pg*LPS characteristics which was both TLR2 and TLR4 ligands. The allergic reaction was measured based on the modulation of allergic reaction biomarkers that was leukotriene C4 (LTC4)[16] which produced by mast cells and basophils; and eosinophilic cationic protein (ECP)[17] which produced by eosinophils in gingival and bronchus tissues. Other examination was IL-4[18] and IgE[19] serum level.

The objective of this study was to verify a new concept that non-allergic children may become allergic after exposed to *P. gingivalis* lipopolysaccharide using Wistar rats as animal model. Additionally, it also verify a new theory of asthma's pathogenesis that allergy is not always inherited but also induced by periodontopathic bacteria such as *P. gingivalis*.

MATERIALS AND METHODS

Twenty four male Wistar rats (120-150 grams) were randomly selected and divided into two experimental groups (A) and one control group (B). Treatment group was divided into 3 subgroups (A1-3) which each consisted of 6 rats. Subgroups 1, 2 and 3 were non allergic rats which subjected to intrasulcular (i.s.) injection of $PgLPS_{1435/1450}$ according Dumitrescu's[20] method to create chronic gingivitis in rats in first day and second day, there were injected one of three doses 0.3, 1.0 and 3.0 μg/mL consecutively (Figure 1). These 3 doses represented low (0.3 μg/mL), cut off point (1,0 μg/mL) and high (3.0 μg/mL).[10] Control group

was injected with phosphate buffered saline (PBS). After 4 days, non-allergic rats were induced by ovalbumin (Sigma-Aldrich, Germany) inhalation that was 1 mg/mL in sterile saline 1 mL dose using nebulizer (OMRON™, USA) for 30 minutes.[21]

Each step (control, fourth day, day 4 after OVA inhalation), tissue samples were taken by sectioning the gingiva and extrapulmonary bronchus (Figure 2), and every time sacrifice was done according to the euthanasia protocol. Blood serum was taken via tail vein during experimental procedures and from the heart after eutahanasia. The allergic reaction was measured based on the modulation of allergic reaction biomarkers that was leukotriene C4 (LTC4)[16] which produced by mast cells and basophils; and eosinophilic cationic protein (ECP)[17] which produced by eosinophils in gingival and bronchus tissues. Allergic reaction biomarkers expressions of gingiva and bronchus immunohistochemistry samples were counted per view with light microscope (Olympus™ CX-31). Other examination was IL-4[18] and IgE[19] serum level. Tissue samples were examined with peroxidase immunohistochemistry using diamino benzidine (DAB), and blood serum with serum enzyme-linked immunosorbent assay ELISA.

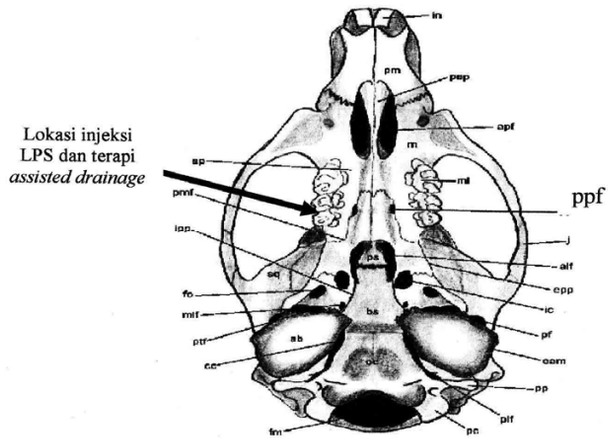

Figure 1. Location of injection and Assisted drainage therapy in Wistar rats.

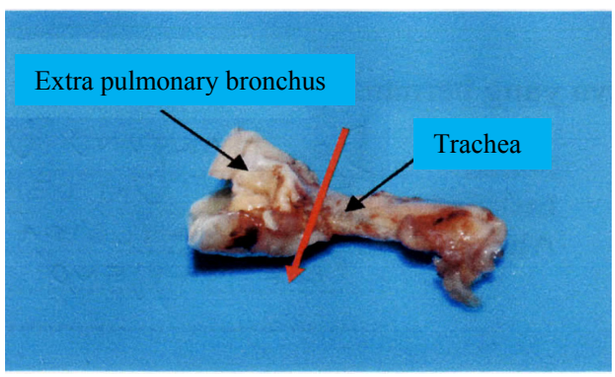

Figure 2. Location of extra-pulmonary bronchus sample section.

This research and its laboratory examinations were conducted in the Biology Department Universitas Brawijaya Malang, October 2008 until February 2009. The research protocol had been approved by the Animal Care and Use Ethical Committee, Faculty of Veterinary Medicine Airlangga University, Surabaya. Statistical analysis was done with ANOVA to reveal the interaction between variable doses *Pg*LPS$_{1435/1450}$ and ovalbumin inhalation.

RESULTS

After intrasulcular injection of *Pg*LPS $_{1435/1450}$ 0.3, 1.0 and 3.0 µg/mL, in day 1 and 2, there were several modulation of allergic reaction biomarkers of gingival tissue and bronchus tissue in day 4. Tissue examination results and statistical significance were shown in Table 1 and 2. The expressions of the allergic reaction biomarkers (LTC4, ECP) after *Pg*LPS $_{1435/1450}$ injection Wistar rats in day 1 and 2. All variables increased significanly in bronchus tissue and insignificant in gingival tissue.

The expressions of the allergic reaction biomarkers (LTC4, ECP) after *Pg*LPS $_{1435/1450}$ injection Wistar rats in day 4 as well as after OVA inhalation. In gingival and bronchus tissues all variables increased significanly, except in bronchus tissue *Pg*LPS$_{1435/1450}$ 1.0 µg/ml. Table 3 showed The serum level after *Pg*LPS $_{1435/1450}$ injections Wistar rats on day 4 all variables were increased significantly except in IL-4 *Pg*LPS$_{1435/1450}$ 0.3 µg/ml.

Table 1. Allergic reaction in non-allergic rats with *Pg*LPS $_{1435/1450}$ injection (gingival and bronchus tissues, day 4)

Dependent variable	Group B Mean ± SD	Group A Mean ± SD					
		0.3 µg/mL n = 6	p	1.0 µg/mL n = 6	p	3.0 µg/mL n = 6	p
Gingiva							
LTC4	2.333 ± 1.211	3.50 ± 0.548	0.520*	3.167 ± 0.983	0.757*	2.333 ± 2.066	1.000*
ECP	3.667 ± 1.632	4.167 ± 1.472	0.590*	4.333 ± 1.366	0.864*	5.167 ± 0.753	0.322*
Bronchus							
LTC4	2.167 ± 0.753	12.833 ± 3.971	0.001	10.667 ± 1.506	0.001	12.333 ± 1.751	0.001
ECP	2.667 ± 1.211	14.167 ± 3.061	0.001	16.50 ± 2.881	0.001	13.333 ± 2.658	0.001

*insignificant difference (p≥.05)

Table 2. Allergic reaction in non-allergic rats with *Pg*LPS $_{1435/1450}$ injection (gingival and bronchus tissues, day 4) pre-inhalation and inhalation

Depend Variable	$PgLPS_{1435/1450}$ 0.3 µg/ml inj.		p	$PgLPS_{1435/1450}$ 1.0 µg/ml inj.		p	$PgLPS_{1435/1450}$ 3.0 µg/ml Inj.		p
	Pre Inhalation	Inhalation		Pre Inhalation	Inhalation		Pre Inhalation	Inhalation	
	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	
Gingiva									
LTC4	3.50 ± 0.548	20.50 ± 0.548	.001	3.167 ± 0.983	24.167 ± 3.764	.001	2.333 ± 2.066	24.67 ± 3.14	.001
ECP	4.167 ± 1.472	22.50 ± 1.643	.001	4.333 ± 1.366	22.00 ± 1.549	.001	5.167 ± 0.753	24.167 ± 1.17	.001
Bronch									
LTC4	12.833 ± 3.971	30.833 ± 1.835	.001	10.667 ± 1.506	26.833 ± 4.021	.001	12.333 ± 1.751	28.833 ± 3.06	.001
ECP	14.167 ± 3.061	25.00 ± 3.225	.001	16.50 ± 2.881	19.50 ± 2.074	.065*	13.333 ± 2.658	29.50 ± 1.049	.001

*insignificant difference (p≥.05)

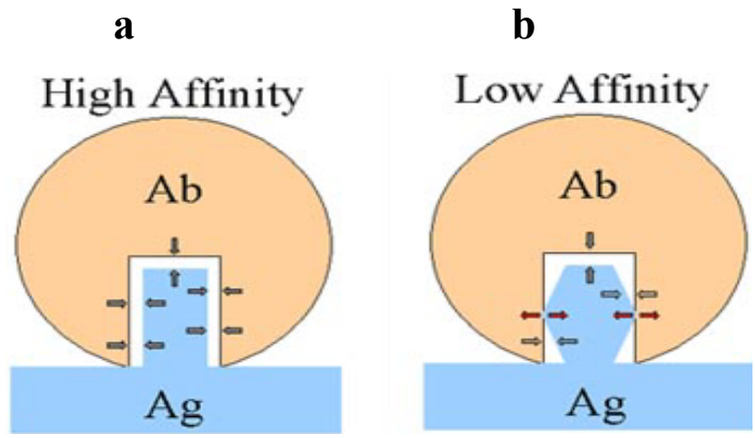

Figure 3. a) Perfect match Ab-Ag; b) Cross-reactivity Ab-Ag.[22]

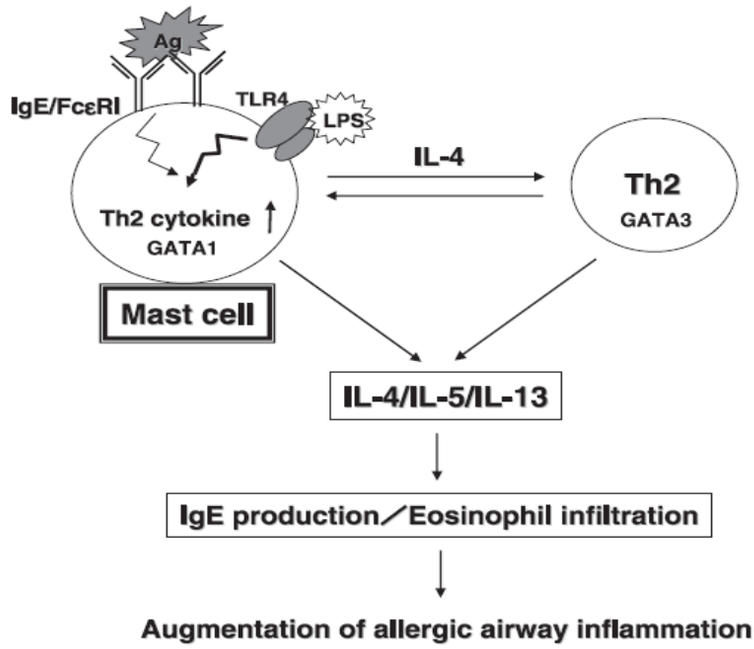

Figure 4. Augmentation of allergic airway inflammation through the TLR4-mediated modification of mast cell.[25]

Table 3. Serum allergic biomarkers in non-allergic rats with *Pg*LPS $_{1435/1450}$ injection (day 4)

Dependent variable	Group B Mean ± SD	Group A Mean ± SD					
Adaptive immune reposnse		0.3 µg/mL n = 6	p	1.0 µg/mL n = 6	p	3.0 µg/mL n = 6	p
IL-4	0.833 ± 0.432	3.983 ± 2.271	0.790*	16.830 ± 8.256	0.001	20.194 ± 6.324	0.001
Allergy IgE	2.482 ± 0.073	8.744 ± 1.874	0.001	9.337 ± 1.799	0.001	7.784 ± 0.902	0.001

*insignificant difference (p≥.05)

DISCUSSION

The classic concept by Strachan in 1989 "the Hygiene Hypothesis" which simply interpreted as "cleanliness makes allergy" was the prime barrier for our concept since it was believed by laymen or by allergic specialist. Our concept so called as "dental plaque-induced asthma hypothesis" was considered controversial before conducting a clinical study. Oral infection such as periodontitis was considered protective to allergic asthma. Despite these obstacles, however, the result of our study was very successful.[5,6]

Dental plaque therapy reduced wheezing, improve respiratory quality based on forced expiratory quality in one second (FEV1) in asthmatic children in one week study.[5] Within the same samples, Utomo and Harsono revealed decreased histamine serum level. Moreover, based on the same study Utomo reported that in randomly selected samples they were still symptomatic after two months later.

Nevertheless, since it was only a clinical study, in order to understand the mechanism a verification study with animal model should be conducted. Utomo revealed that allergic asthma symptoms which caused by immunogenic as well as neurogenic inflammation also increased after injected with *Pg*LPS$_{1435/1450}$. Interaction of these inflammations increased allergic symptoms even more. Therefore,it is possible that *Pg*LPS is a trigger of allergic reaction in non-allergic rats.[27]

A concept which proposed by Yoo *et al*. in 2007 attracted us to reveal why non-allergic asthma children could become allergic eventhough not inherited by their parents, Therefore, an animal study of non-allergic rats which induced for chronic gingivitis with *Pg*LPS$_{1435/1450}$ as well as evaluating the allergic reaction was conducted. After *Pg*LPS$_{1435/1450}$ injection in non-allergic rats, in day 4 examination revealed that in gingival tissue ECP expression increase significantly in all doses (p=0.003; p=0.006 and p=0.001). Nevertheless, LTC4 expressions also increased eventhough insignificant (Table 1). In extrapulmonary bronchus, LTC4 and ECP expressions increased significantly in all doses (p=0.001 in all variables). The increased expressions could be via the activation of toll-like receptor-2 (TLR2) and/or TLR4 in immunocompetent cells after *Pg*LPS$_{1435/1450}$ injection, which then released mediators.

Ovalbumin inhalation for 30 mins, as predicted did not change systemic immun response, since no significant change of IL-4 and IgE serum level (Table not included). Nevertheless, interestingly, Ovalbumin inhalation resulted in significant increase of LTC4 and ECP expressions in gingival and bronchus tissues (p=0.001), except ECP expression in bronchus 1,0 µg/mL *Pg*LPS$_{1435/1450}$ injection (p=0.065) (Table 2). It was logical since according to literatures OVA inhalation increased LTC4[16] and ECP[17] in OVA-induced allergic rats. Neverheless, this result was "out of the box" since without any previous OVA-induced allergy injection, allergic reaction also occured. This phenomenon was considered the hardest thing to explain since it was an unusual finding.

In a simple explanation, OVA inhalation acts as specific allergen which cross-linked with the OVA-specific IgEs that attached to mast cells and basophils in OVA-induced allergy subjects and stimulated degranulation. Nevertheless, even without previous OVA-induced allergic sensitization, antigen-antibody reaction also happened, this mechanism is termed as cross reactivity.[22] According to Melton and Landry,[23] cross reactivity may happens if a protein which actually has a 3D shape, in this case the Ovalbumin inhalation in our study, could attach to the non-specific IgEs even not perfect match as "lock and key". (Figure 3), in our study non-OVA spesific IgE recognized OVA as its ligand and activate mast cells.

The result of our study is supported by Saluja *et al*.[24] study in 2012, which reported that prolonged exposure (96 h) with TLR-ligands promoted mast cell reactivity following IgE-receptor activation. TLR4 activation with LPS generated the most pronounced effect, with an enhanced degranulation and secretion of leukotrienes, cytokines and chemokines. The effect of LPS was mediated through a Myd88-dependent pathway and the increased effect involved JNK-dependent pathway. In our study, this mechanism was suggested via FceR1-mediated mast cell reactivity amplification after stimulation of TLR2 and -4 with *Pg*LPS$_{1435/1450}$ (Figure 4). This mechanism was also confirmed in this study, after *Pg*LPS$_{1435/1450}$ injection TLR2 and -4 expressions in rats' bronchus were higher significantly (p=0.001) compared to control (Table not included).

There is an important question:"Which cells are the first target of *Pg*LPS injection as in our study?" It is not

easy to answer, but it will answer our research question. Mast cells has a strategic location in the body; they are common at sites in the body that are exposed to the external environment, such as mucosa and the skin. In these locations, they are found in close proximity to blood vessels, where they can regulate vascular permeability and effector-cell recruitment.[25] Although they do not have direct cell to cell contact with local populations of antigen-presenting cells, mast cells can modulate the behaviour of these and other neighbouring effector cells through the release of mediators.[26]

According to Kulka *et al.*,[27] mast cells products such as histamine and tryptase stimulates nerve endings, thus neurogenic inflammation, which then secretes neuropeptides such as substance P or calcitonin gene-related peptide. These neuropeptides activates either basophils or mast cells via neurokinin recepters which then conducting a "vicious circle" that aggravated allergic reaction. Berry *et al*.[28] reported that these cells also secretes TNF-α which able to complicate asthma pathogenesis which termed as refractory or "difficult asthma". Moreover, these cells also produced IL-4 that needed for isotype switching mechanism from IgG or IgM to IgE, thus also increased IgE in serum level. This mechanism was verified by the increase of IL-4 serum level which was significant (p=0.001) in 1.0 and 3,0 µg/mL as well as an increase but insignificant (p=0.790) in 0.3 µg/mL (Table 3).

The presence of other specific IgEs which not induced by OVA sensitization in this study also supported by Hahn *et al.*[29] study in 2012 which reported that *Chlamydia pneumonia*-specific IgE is prevalent in asthma and associated with asthma severity. Therefore, there must be "still undiscovered" specific-IgE which produced after *Pg*LPS injection. The presence of this kind of IgEs may resulted to cross-reativity, thus increased mast cells or basophils activation which lead to increase allergic reaction.

Our study supported the successful dental plaque control therapy in reducing allergic asthma symptoms i.e. wheezing in Wiyarni *et al*.[6] study. In this study, asthmatic children who had been conducted dental plaque control therapy and Dental Health Education had lower positive bacterial culture of Gram negative bacteria significantly compared to control in o week study. Moreover, a longer evaluation, that was until two month evalution, of randomly selected samples of this clinical study revealed that the allergic asthma symptoms of those children were still disappeared.[7]

It was not surprising since free specific IgE (free/unbounded) actually present for 1-2 days only, compared to 21 days if attached to mast cells or basophils, after that it would be degraded.[30] Consequently, decreasing allergic reaction by reducing the activity of mast cells and basophils that were stimulated by LPS and neuropeptides is a better way than considering the IgE level only. It was supported by Gamble *et al*.[31] in 2010, who reported that specific IgE was not the best biologic predictor for allergic asthma severity, since it was racial dependent. The diminished activation of mast cells and basophils resulted in lowering interleukin-3 (IL-3) excretion that functions as stimulator of mast cell proliferation and basophils apoptosis prevention.[30]

Based on this study, it can be concluded that even in non-allergic individuals, a prolonged exposure of *Pg*LPS is able to elicit allergic reaction, enhancing cross-reactivity and increase FcεRI expressions, thus mast cells and basophils reactivity which lead to increase allergic symptoms. Therefore, eliminating periodontopathic bacteria is mandatory to prevent from allergy and allergic transmision.

ACKNOWLEDGEMENT

Promotor and co-promotors of the Doctorate Program in Universitas Airlangga: Prof. Subijanto Marto Sudarmo, MD., Ph.D, Pediatric Gastroenterology Consultant; Prof. Fedik Abdul Rantam, DVM., Ph.D. and Prof. M. Rubianto, DDS., Ph.D, Periodontist Consultant.

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