Immuogenic Potency of LPS Actinobacillus Actinomycetemcomitans Local Isolate on Iga, Siga, and Igg Titre in Aggressive Periodontitis

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Abstract

The incidence of periodontitis which is varied in different countries around the world shows an increasing tendency in lipopolysaccharide (LPS) of Actinobacillus actinomycetemcomitans which is part of the cell wall of one of the virulence factors. The purpose of this study is to obtain and to characterize LPS of Surabaya isolates A. actinomycetemcomitans as the main cause of aggressive periodontitis and and to analyze the effect of LPS on the induction of IgA and IgG titers in serum and saliva.

The method used in this study uses phenol to obtain and to characterize and purification LPS A. actinomycetemcomitans. Furthermore, it conducted in experimental animals (Wistar rats). This is done by calculating the levels of IgA, sIgA, and IgG with ELISA technique. This study results LPS of A. actinomycetemcomitans which is in accordance with LPS of E. coli O127 which is the standard of LPS. In experimental animals, the results show that the level of IgA in serum has increased although there is no significant difference with the control group from the statistical test, whereas the level of IgA in saliva shows significant differences if they are compared from the control group and the treatment group. The level of IgG in the treatment group also seems increasing although the statistical analysis did not show any significant difference from the controlled group.

LPS of A. actinomycetemcomitans can increase the antibody of Wistar rats, especially the level of IgA in saliva (sIgA). This suggests that sIgA roles in local immune defense.

Experimental article (J Int Dent Med Res 2017; 10: (1), pp. 128-133)

Keywords: Aggressive periodontitis, IgA, IgG, LPS A. actinomycetemcomitans, sIgA.

Received date: 16 January 2017 **Accept date:** 05 February 2017

Introduction

Progressive periodontitis prevalence in the clinic of RSGM FKG Universitas Airlangga Surabaya and the internal poly of RSU DR Soetomo has been increasing. Existing data in 1991 by 9% became 23% in 2003. Observations conducted in January-December 2006 showed that there were 288 patients with periodontitis where 57 of them are people with aggressive periodontitis.

The pathogenesis of periodontitis is influenced by the interaction between the host and microbial factors dominated by *A. actinomycetemcomitans*. The presence of these bacteria in dental plaque is caused by aggressive periodontal destruction and aggravated by the presence of genetic and environmental factors.[1] Lipopolisakarida (LPS) is part of the cell wall which is one of the virulence factors of A. actinomycetemcomitans. This LPS will interact with the surface receptors of epithelial cell via serum proteins. The increasing LPS will boost the production of IL-1β and IL-6 that can cause damage to the periodontal tissues because LPS has potency to stimulate cytokine responses in epithelial, neutrophils, fibroblasts and monocytes in periodontal tissues. LPS activates monocytes, macrophages and fibroblasts that produce proinflammatory cytokines, namely IL-1β, IL-6 and TNF-α. These cytokines will stimulate MMPs, ie MMP-1, MMP-3 and MMP-13 collagenase, which will damage the tissue through the degradation of extracellular matrix components. IL-1 and TNF-α will reabsorb bone by stimulating IL-6 indirectly or by stimulating directly effector related to osteoclastogenesis NFκB like receptor ligand (RANKL). LPS may play a major role in increasing osteoblastic expression of

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prostaglandin E2, RANKL, IL-1 and TNF-α. The existence of A.actinomycetemcomitans will increase RANKL expression in CD4 + cells that would activate osteoclasts thereby increasing alveolar bone destruction.[2]

Humoral immune responses have a protective role in the pathogenesis of periodontitis. Changes in the specific response of IgG and IgA locally and systemically have relevance progression of the disease.[3] The results of the research done by Lakio et al indicate the increasing of plasma IgG on bacterial pathogens in periodontal tissues, especially in patients with aggressive periodontitis.[4] *Salivary* IgA (sIgA) is the predominant immunoglobulin saliva and roles in local immune defense system.

The measurement of plasma and salivary antibodies is used to diagnose periodontitis, to estimate activity, to have classification and prognosis, and to indicate treatment success.[4]

Materials and methods

Creating culture of Actinobacillus actinomycetemcomitans

From the stock of bacteria A. actinomycetemcomitans, replanting is done on Luria Berthani media for 2-3 days so the morphology picture of bacteria emerges (made in 2 plates). Gram's staining is done and planted in BHI broth medium so the culture stickly emerges in tube wall.

The isolation and the purification of crude LPS Actinobacillus actinomycetemcomitans

The isolation of crude LPS A.actinomycetemcomitans is done by using the method of Westphal and Jann taken from Westerman, 1977. Bacteria is planted in Luria broth medium containing yeast extract and triptone, then it is incubated at 37 ºC for 16-18 hours. 500 ml of bacterial suspension are centrifuged with 15000 g (6000 rpm) for 1 hour. The obtained pellet is resuspended in 10 ml *deionized water* (which has been heated at 72-75 ºC), then it is vortexed for 20 seconds and placed in a water bath at 72-75 ºC with open mouth tube. Next, 10 ml of 88% phenol solution are added (previously heated at 72-75 ºC), then the tube is vortexed and incubated for 15 minutes in *waterbath.* The tube is revortexed for certain minutes during incubation, then it is frozen for 15 minutes at room temperature. After 500 g (100 rpm) centrifugation for 10 minutes, the water phase put in the upper layer is moved and saved. The middle layer, which is the phenol phase, is heated at 72-75 ºC, then 10 ml deionized water are added. Furthermore, the procedure is replied. The water phase from the twice extraction procedure is mixed and heated at 72-75 ºC. 5 ml of 88% phenol (previously heated at 72-75 ºC) are added, and that mixture is incubated for 15 minutes (at 72-75 ºC) by which vortex is done periodically and replying extraction process. The middle layer containing precipitated protein is discarded. The existence of the phenol phase is proved by the 15000 g (6000 rpm) centrifugation for 20 minutes. The water phase is dialyzed in water (at 4 ºC) until all the remain phenol are discarded. Lipopolisakarida is precipitated from the water phase with 95% ethanol (containing 0.15 per 50 ml of sodium acetate) 6 times overnight at -20 ºC. The pellet is resuspended in 1 ml of water and saved at -20 ºC.

For purification, *crude* LPS is done by filtrating gel Sephadex C-18 at room temperature with disaggregation buffer as (0.05 M Tris-HCl (pH 9), 0.001 M EDTA, 0.3 *deoxycolate*) solution. Fraction containing LPS is identified with staining with silver nitrate and precipitated by adding 0.15 M NaCl and 4 times volume of 95% ethanol. Next, the precipitation results are isolated with 12000 g centrifugation for 20 minutes at 4 ºC, then the pellet is dissolved and dialyzed in water to have lyophilization. Fraction with high molecular weight is observed with

For the purification of crude LPS performed by gel filtration on Sephadex C-18 at room temperature with disaggregation buffer (0:05 M Tris-HCl (pH 9), 0.001 M EDTA, 0.3 deoxycholates) as a solution. Fractions containing LPS identified by staining with silver nitrate and precipitated by adding 0:15 M NaCl and 4 times the volume of 95% ethanol. Results of precipitation are then isolated by centrifugation at 12000 g for 20 min at 4 ° C, then diluted with water and the pellets in the water and lyophilization. High molecular weight fraction was observed by column chromatography Sephadex C-18.[5]

The total calculation of LPS A. actinomycetemcomitans

The level of LPS *A. actinomycetemcomitans* obtained based on the calculation of Biuret standard curve is 3.02 mg/mL = 3.020 ug/mL. To make 200 ug/mL

concentration of LPS *A. actinomycetemcomitans* V1. M1 = V2. M2 calculation is done, so to make 200 ug/mL concentration of LPS *A. actinomycetemcomitans* for 66.2 µL 1000 ml PBS is added. Then, it is suspended by *Complete Freud Adjuvant* (CFA) or *Incomplete Freud Adjuvant* (IFA) by comparison 1:1, and that suspension is ready to be injected to Wistar rats.

Moreover, LPS *A. actinomycetemcomitans* is compared to raw LPS *E.coli* O127 produced by Sigma. The isolation result of LPS *A. actinomycetemcomitans* using spectrophotometer with the standard of LPS *E.coli* O127 indicates the similarities of LPS *A. actinomycetemcomitans* and LPS *E.coli* O127.

The induction of LPS A. actinomycetemcomitans on Wistar rats

Wistar rats are divided into 2 groups, each of group consists of 10 rats. Group 1 is the group controlled with NaCl 0.9%, group 2 is the group treated by injection intraperitoneally (ip) with LPS. The giving of LPS or *A. actinomycetemcomitans* for 200 µg consists of 100 µg LPS or *A. actinomycetemcomitans* and 100 µg *adjuvant.* In the first injection, *Complete Adjuvan* is given, and in the second to the fourth injection, *Incomplete Adjuvan* is given. In the fifth week, blood sampling is done. To obtain enough volume, blood sampling can be done directly from the hearts of rats. Then, ELISA examination is conducted to determine the humoral immune response by determining the levels of IgG and IgA.

Saliva sampling of Wistar rats

Saliva sampling on experimental animals is done by using periodontal *paper* which placing periodontal *paper* on the area under the tongue of Wistar rats. After that, wait until pervasive saliva on that periodontal *paper* reaches certain limit in accordance with periodontal *paper* sign. Then, periodontal *paper* is included in the PBS to be examined by ELISA.

The examination of the levels of IgA and IgG by using ELISA technique

In this research, ELISA method is used to examine the levels of IgG and IgA through following ways: After kit is excluded from 2-8 °C, kit is allowed to stand for 30 minutes at room tempertature. All reagen is prepared before the procedure stage is started. It is suggested that all standard sample is included into the copy of *microelisa stripplate.* The next step is diluting 20X wash solution into 1x wash solution with ddH2O. In setting *well* standard, samples are tested in plate measurement then added 50 ul in *well* standard and 50 ul samples which had been diluted in *well* samples (10 ul samples + 40 ul samples solvent). In *well blanko*, standard solvent is added. Moreover, 50 ul HRP-*conjugate* antibody are added in each *well* except *well blanko.* Next, it is homogenized by shaking slowly and incubating for 60 minutes at 37 °C. After throwing as much as solution, fill the *well* with *washing solution.* Homogenized with *shaker* for 1 minute, the next step is wasting the *washing solution* and removing the remain liquid with filter paper. This procedure could be replied 4 times so the total washing is 5 times. The substrate A and B are added respectively 50 ul in each *well* to be homogenized slowly in incubation for 15 minutes at 37 ºC. Moreover, 50 ul *stop solution* are needed to stop the reaction signed by the changing from blue to yellow. The *optical density* measurement in the 450 nm wavelength for 15 minutes. Then, the standard curve is made with *optical density* as the Y axis and concentrated on the X axis and calculated with the linear regression equation. Therefore, the concentration of the samples is known.

Results

The Purification of LPS A. actinomycetemcomitans

Figure 1. The purification result of LPS *A.actinomycetemcomitans* with Sephadex C-18.

From the obtained isolation result of LPS *A. actinomycetemcomitans,* the purification is conducted by using column chromatography Sephadex C-18, it could be seen in Figure 1.

The isolation and the characterization of LPS A. actinomycetemcomitans

The isolation and characterization results show the similarities between LPS *A. actinomycetemcomitans* and LPS *E.coli.* The examination result using spectrophotometer of LPS *A. actinomycetemcomitans* and *E.coli* could be seen in Figure 2. From Figure 2, it seems that the maximum wavelength to detect both LPS is similar, which is 200 nm and seems similar to the absorbance curve profile for the same wavelength. The purification result of LPS *A. actinomycetemcomitans* is used to induce experimental animas intra peritoneally (*i.p*.)

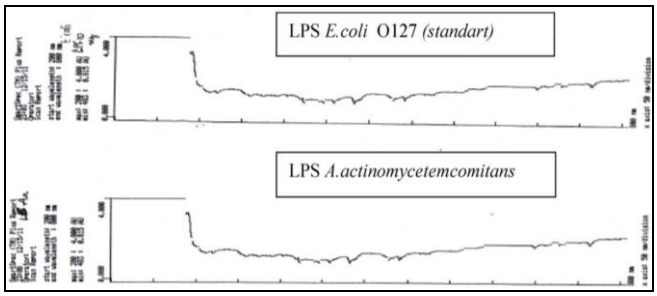

Figure 2. The examination result of LPS *A. actinomycetemcomitans* using spectrophotometer with 200 nm wavelength.

The level of IgA in serum

The statistical analysis on different test of the level of IgA in serum between controlled groups induced by NaCl 0.9% and treated groups induced by LPS *A. actinomycetemcomitans* is conducted by using *T test* since the result of normality examination using *Shapiro-Wilk test* shows the normal data distribution ($p > 0.05$) and its homogen variance shown from *levene's test* result ($p > 0.05$). The statistical analysis result using *T test* indicates the level of IgA in serum between controlled groups induced by NaCl 0.9% and treated groups induced by LPS *A. actinomycetemcomitans* has no significant difference ($p = 0.262$), but the average level of IgA in serum at treated groups induced by LPS *A. actinomycetemcomitans* is higher than controlled groups induced by NaCl 0.9% (Table 1).

The level of IgA in saliva (sIgA)

The statistical analysis on different test of the level of sIgA in saliva (sIgA) between controlled groups induced by NaCl 0.9% and treated groups induced by LPS *A. actinomycetemcomitans* is conducted by using *T test* since the result of normality examination using *Shapiro-Wilk test* shows the normal data distribution ($p > 0.05$) and its homogen variance shown from *levene's test* result ($p > 0.05$). The statistical analysis result using *T test* indicates the level of IgA in serum between controlled groups induced by NaCl 0.9% and treated groups induced by LPS *A. actinomycetemcomitans* has significant difference ($p = 0.026$) by which the average level of sIgA treated groups induced LPS *A. actinomycetemcomitans* is higher than controlled groups induced by NaCl 0.9% (Table 2).

Groups	X	SD	Min	Max	T test
Controlled	2510.06	925.76	1212.60	4219.91	F = 0.273
LPS *A.actinomycetemcomitans*	3036.89	1101.98	1839.41	5081.76	p = 0.262

Table 1. The average and the standard deviations of the level of IgA in serum towards LPS *A.actinomycetemcomitans.*

Groups	X	SD	Min	Max	T test
Controlled	344.04	231.08	108.13	931.77	F = 0.138
LPS *A.actinomycetemcomitans*	557.78	157.34	320.37	766.87	p = 0.026

Table 2. The average and the standard deviations of the level of IgA in saliva (sIgA) towards *A.actinomycetemcomitans.*

Groups	X	SD	Min	Max	T test
Controlled	7335.22	3083.56	4075.53	12819.16	F = 0.552
LPS *A.actinomycetemcomitans*	8970.97	2829.49	3738.18	12380.38	p = 0.232

Table 3. The average and the standard deviations of the level of IgG in serum towards *A.actinomycetemcomitans.*

The level of IgG in serum

The statistical analysis on different test of the level of IgG in serum between controlled groups induced by NaCl 0.9% and treated groups induced by LPS *A. actinomycetemcomitans* is conducted by using *T test* since the result of normality examination using *Shapiro-Wilk test* shows the normal data distribution ($p > 0.05$) and its homogen variance shown from *levene's test* result ($p > 0.05$). The statistical analysis result using *T test* indicates the level of IgG in serum between controlled groups induced by NaCl 0.9% and treated groups induced by LPS *A. actinomycetemcomitans* has no significant difference ($p = 0.232$), but the average level of

IgG in serum at treated groups induced by LPS *A. actinomycetemcomitans* is higher than controlled groups induced by NaCl 0.9% (Table 3).

Discussion

The calculation result of the level of IgA indicates that the average level of IgA of treated groups induced by LPS *A. actinomycetemcomitans* is higher than controlled groups induced by NaCl 0.9% (Figure 3). Although there is no significant difference among them, it shows that LPS is an immunodominant surface antigen of *A. actinomycetemcomitans.* As the main component of outer membrane of bacteria *A. actinomycetemcomitans,* LPS roles as microbial molecular patterns associated with receptors of pattern recognition on immune and non-immune cells in periodontal tissues. The molecules of LPS *A. actinomycetemcomitans* are very active in biological system and they are able to have inflammatory manifestation and to destroy this disease.[6] The specific antibody system, including IgA, founded in saliva is complex body fluids to help the cleaning process used to early diagnose and to detect potential susceptibility to some diseases.[7]

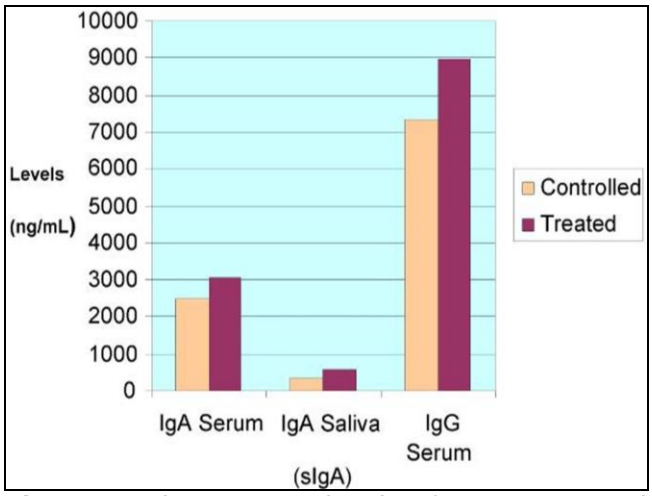

Figure 3. The average levels of IgA, sIgA and IgG.

The calculation result of the level of sIgA indicates that the average level of sIgA of treated groups induced by LPS *A. actinomycetemcomitans* is higher than controlled groups induced by NaCl 0.9% and has significant difference among those groups. It shows that sIgA is the first important defensive line against pathogens which attacks mucosal surfaces of the oral cavity. Several previous research confirm the existence of the protection and the role of sIgA in patients with periodontal disease, and the low concentration of sIgA has been associated with the severity of the disease. sIgA is the dominant immunoglobulin secreted by oral mucosa and has been considered as the main factor which gives contribution on mucosal health and defense against microbes. The total IgA in saliva is the mixture of dimeric secretory of IgA derived from plasma cells in the salivary glands and monomeric of IgA derived from plasma in the gingival sulcus. Therefore, the comprehensive analysis of saliva is a diagnostic device. The overall changing on saliva composition could help to understand the severity increase of periodontal disease and to diagnose the possible variations and the defective immune response including sIgA secretion.

The calculation result of the level of IgG indicates that the average level of IgG of treated groups induced by LPS *A. actinomycetemcomitans* is higher than controlled groups induced by NaCl 0.9%. Although there is no significant difference among them, this fact is in accordance with the previous research done by Wilson and Hamilton in 1992. The titers of IgG patients with *Localized Juvenile Periodontitis* (LJP) will increase, this fact is caused by LPS *A. actinomycetemcomitans.* Besides the increasing titers of IgG, this fact is also followed by the increasing concentration of IgG and IgG_2 in serum.

LPS *A. actinomycetemcomitans* stimulates the main antibody production of IgG derived from the subclass of IgG_2. This fact is consistent to several other research which indicate that in general polysaccharide antigen is very special since it induces IgG_1 dan IgG_2. Immunoglobulin has effects on microbes in the mouth for it roles on the defense, and through bacterial metabolism, sIgA, IgG , and IgM have higher concentration on patients with periodontal disease than healthy patients.[8]

Conclusions

Based on the results of this research, it could be concluded that the research successfully produce LPS *A. actinomycetemcomitans* which is in accordance with LPS *E. coli* O127 (the standard of LPS).

Moreover, the research has been proven that LPS *A. actinomycetemcomitans* could increase the levels of IgA, sIgA and IgG on experimental animals (Wistar rats). To sum up, further research is needed to clarify the character of *LPS of Surabaya isolates A. actinomycetemcomitans.*

Acknowledgements

The author thank to the Airlangga University for facilitating this research. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of Interest

The authors report no conflict of interest and the article is not funded or supported by any research grant.

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