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An ELISA-based detection of *Porphyromonas gingivalis* in the dental plaques of periodontal diseased patients*)

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Abstract

Murine hybridomas producing lipopolisaccharides of Porphyromonas gingivalis *(LPS-Pg)-specific monoclonal antibodies were established. Dental plaques were obtained from periodontal healthy subjects and patients with gingivitis or periodontitis. The levels of* P. gingivalis *were semiquantitatively detected by ELISA. The results showed that antibodies produced by clone Pg.C.5 were lgG2a and recognized specifically LPS-Pg, but not LPS from other periodontopathic or enteric bacteria. The presence of* P. gingivalis *in the dental plaque of periodontal diseased patients was able to be detected by ELISA using these antibodies. The levels of this bacterium in patients with periodontitis were much higher than those of patients with gingivitis or periodontal healthy subjects ($p<0.01$), indicating that bacterial monitoring for the presence of* P. gingivalis *in the ental plaque may be carried out semiquantitatively by ELISA and may differentiate the early (gingivitis) and progressive (periodontitis) periodontal disease.*

Keywords: Porphyromonas gingivalis – *ELISA* – *periodontal disease*

Introduction

Chronic inflammatory periodontal disease (CIPD) is an inflammation of periodontal tissues following pathogenic activities of dental plaque periodontopathic bacteria (Christersson *et al.*, 1991). Along with gram negative bacteria such as *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis* is one of the predominant bacteria isolated from dental plaque of periodontal diseased patients (Kamma *et al.*, 1994 and 1995; Darveau *et al.*, 1997). Increased number of this black pigmented microorganis is significally associated with increased gingival pocket dept at periodontal diseased sites (Kamma *et al.*, 1994; Kigure *et al.*, 1995), suggesting that this type of bacteria plays a crucial role in the course of CIPD. Indeed, increased immune responses such as increased *P. gingivalis*-specific serum antibodies and -specific gingival and peripheral blood mononuclear cell activation in patients with CIPD certainly support the role of this bacteria in the progression of this CIPD (Sosroseno and Herminajeng, 1995; Ishikawa *et al.*, 1997). Lipopolysaccharide (LPS) derived from this

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bacterium as one of the most potent antigens in the course of CIPD has been well documented. The abilities of LPS of *P. gingivalis* (LPS-Pg) to induce alveolar bone destruction (Nair *et al.*, 1996) and to act as a potent mitogen for B cells producing polyclonal antibodies (Ishikawa *et al.*, 1997) are only examples of biological properties of LPS-Pg, showing the virulence of this bacterium on periodontal tissues.

Detection of dental plaque P. *gingivalis* using various microbial detection methods to delineate the pathogenesis of as well as to establish clinical diagnosis of CIPD has been carried out. BANA test (Amalfitano *et al.*, 1993), and routine bacterial cultures (Kamma *et al.*, 1994 and 1995) have been applied; as yet, they tend to be labored ones. Advanced and rapid tests using bacterial specific DNA probes have also been developed (Melvin *et al.*, 1994; Wong *et al.*, 1906). Although these very powerful methods may distinguish bacterial polymorphisms, drawbacks of these molecular approaches to the developing countries seem to be expensive and it requires highly trained operators as well as specific facilities. Fluorescence or enzyme-labelled *P. gingivalis*-specific antibodies have further been utilized to detect *P. gingivalis* (Wolff *et al.*, 1992; Melvin *et al.*, 1994). The aims of this study was to establish murine monoclonal antibodies specific to *P. gingivalis*-derived lipopolysaccharides (LPS-Pg) and to semiquanitatively detect dental plaque *P. gingivalis* isolated from periodontal healthy subjects and patients with gingivitis or periodontitis by an ELISA method.

Materials and Methods

Antigens

Lipopolysacchrides (LPS) derived from P. *gingivalis*, *Actinomycetemcomitans*, *Fusobacterium nucleatum* and *Provotella intermedia* were generously gifted by Prof. Greg Seymour. LPS from *Salmonella thyposa* was commercially obtained (Sigma, USA).

Production of monoclonal antibodies

Murine monoclonal antibodies specific to P. *gingivalis* were produced as described elsewhere (Bird and Seymour, 1987). Briefly, female 6-8 week old Balb/c mice were intraperitoneally injected with LPS solution (100 mg/100 ul PBS) one per week for 3 weeks. Mice were killed by asphyxiation and spleen cell suspension obtained aseptically. Spleen cells ($1X10^6$ cells/ml/well) were cultured in 24 well plates in RPMI medium 1640 (Sigma, USA) supplemented with 10 foetal calf serum (Gibco) and 1% antibiotics (Gibco) in an incubator with 5% $C0_2$ and temperature of 37°C. Following stimulation with LPS-Pg (0.5 ug/well) for 2 days, fusion of splenic cells and murine myeloma cells (NS2 cells) was carried out in HAT medium (Sigma, USA). Following identification of cell growth, cells were harvested. Limited dilution was done to obtain one cell per well. Feeder cells obtained from mouse peritoneal cell were added to the plates and after 3-4 day incubation, the cultures were harvested. Identified cells were then recultured and added with feeder cells.

Characterization of monoclonal antibodies

Culture supernatans were harvested and partially purified by aluminium sulfate. Antibody classes and subclasses were checked by ELISA (Bird and Seymour, 1987; Sosroseno, 1992). Preliminary data showed that these antibodies were able to recognize efficiently LPS-Pg by ELISA (data not shown). In these experiments, ELISA plates (Nuncl) were coated with 50 ul of antigen solution containing various concentrations of LPS-Pg. Partially purified antibodies, diluted in 1:10, were added. Following washing, biotin labeled-sheep anti-mouse Ig (Sigma, USA), diluted in 1:1000, was then added and following incubation, peroxidase-streptavidine, diluted 1:10.000 was added. Colour was developed by adding TMB solution (Sigma, USA) as colour substrate and the reaction was stopped after 10 minutes with H_2SO_4. Colour was then read with an ELISA reader (Flow Laboratorium) at a wavelength of 450 nm. In order to determine antibody isotypes, ELISA was carried out as above. Plates were coated with 0.5 ug of LPS-Pg in 50 ul of PBS per well. Except that secondary antibodies were biotin labeled-sheep anti-mouse IgG, IgA, IgM, IgG, IgG1, IgG2a, IgG2b and IgG3 (Sigma, USA). The sheep anti-mouse IgM was diluted in 1:5000, whereas all other antibodies were diluted in 1:1000. Antigen specificity was done by ELISA as above. In this respect, plates were coated with 50 ul antigen solution containing 0.5 ug of LPS-Pg or LPS of *A. actinomyucetemcomitans*, *F. nucleatum*, *P. intermedia* or *S. thyposa*. The secondary antibodies were biotin labeled-sheep anti-mouse Ig (Sigma, USA), diluted in 1:1000.

Preparation of dental plaque samples

Subjects were obtained from Clinic of Periodontology, Faculty of Dentistry, Gadjah Mada University, Indonesia, and all were checked for dental plaque score, dental calculkus scores, gingival pocket depth, spontaneous gingival bleeding, alveolar bone destruction by Ro photos. Based upon clinical criteria, subjects were divided into 3 groups, i.e., periodontal healthy subjects, and patients with gingivitis or periodontitis, and were briefly informed the aims of the study for obtaining an agreement. Dental plaques were removed by a sterile dental scalier and immediately put into 5 ml tubes, each containing 0.5 ml steril PBS. Tubes were kept in -20°C until used.

Dental plaque bacterial detection by ELISA

Dental plaques were diluted in 1:10 in steril PBS and was coated onto the ELISA plates. In each plate, coating with LPS-Pg and PBS was done for positive and negative internal controls respectively. Following incubation at room temperature and washing for 3 times, diluted monoclonal antibodies anti-LPS-Pg (1:100) were then added. The remaining ELISA protocols were as described above.

Results

Characterizations of monoclonal antibodies specific to LPS-*P. gingivalis*

Fusion of splenic cells of LPS-Pg-immunized mice and NS2 myeloma cells resulted in few hybridoma cells. Following limited dilutions, clone Pg.C.5 out of few clones was chosen to be characterized, as it seemed to grow well. First characterization determined the antibody classes of clone Pg.C.5 culture supernatans. Antibodies were only recognized by anti-mouse IgG but not IgA and IgM, suggesting that antibodies produced by this clone was IgG ones (Fig. 1). Further characterization was carried out to assess IgG subclasses of these antibodies. Diluted samples were added to antigen-coated plates. Following incubation and washing, biotin labelled-anti-mouse IgG subclass antibodies were added. Only labelled anti-IgG2a antibodies that had been added showed positive results, suggesting that clone Pg.C.S produced IgG2a antibodies (Fig. 1). It is now clear, moreover, that even monoclonal antibodies produced specifically to a given antigen, cross reactions remain to occur due to the fact that closely related antigen epitops may be recognized by the same antibodies. This study utilized LPS from several periodontopathic bacteria such as *F. nucleatum*,

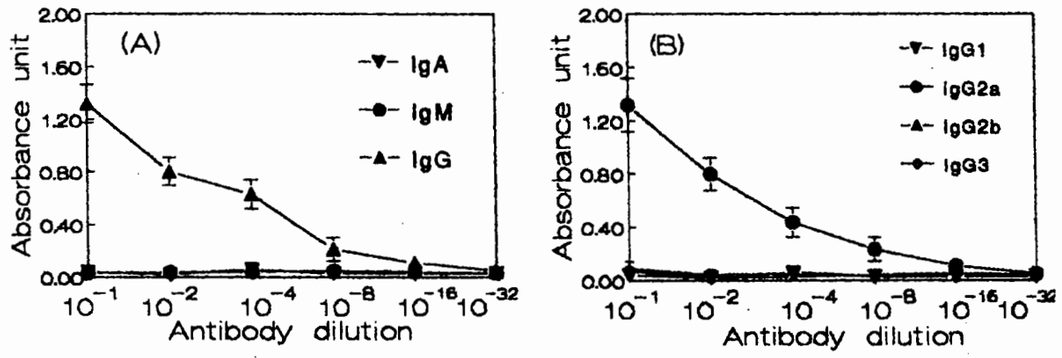

Figure 1. Antibody classes (A) and subclasses (B) produced by hybridomas clone Pg.C.5. Culture supernatant-partially purified antibody classes and subclasses were determined by ELISA

A. actinomycetemcomitans, and *P. intemedia* as well as LPS from unrelated bacteria, i.e., *S. thyposa*. The results showed that antibodies of clone Pg.C.5., only recognized LPS-Pg, but not other tested periodontopathic or unrelated bacteria, indicating that no cross reaction occurred (Fig. 2).

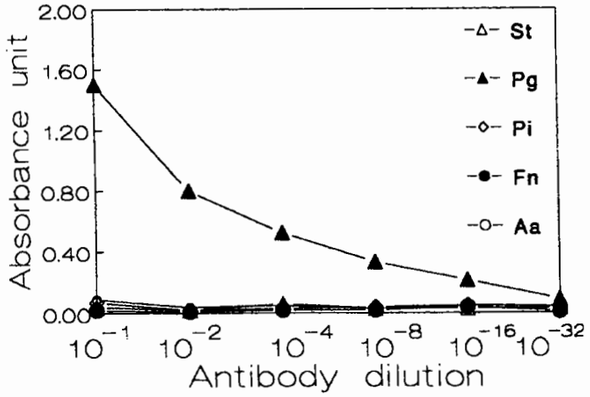

Figure 2. Antigen specificity of antibodies produced by hybridomas clone Pg.C.5. St = LPS of *Slamonella thyposa*; Pg = LPS of *P. gingivalis*; Pi = LPS of *Prevotella intermedia*; Fn = LPS of Fusobacterium nucleatum; Aa = LPS of *Actinobacillus antinomycetemcimotans*.

Detection of dental plaque *P. gingivalis* by ELISA

The role of *P. gingivalis* in the course of progresive periodontal disease and increased number of this bacterium on the dental plaque of patients with this periodontal lesion have been well documented. It would be of interest to apply the antibodies produced by clone Pg.C.5. to detect *P. gingivalis* in dental plaques of periodontal diseased patients. Fifty subjects consisting of 15 of periodontal healthy ones, 17 of patients with gingivitis and 18 of patients with peridontitis were obtained from patients registered in the clinic of Faculty of Dentistry, Gadjah Mada University. Dental plaques were removed from these subjects and coated onto the ELISA plates. Levels of *P. gingivalis* were assessed semiquantitatively by LPS-Pg-specific monoclonal antibodies produced by clone Pg.C.5. Mean of absorbance unit of samples taken from periodontal healthy subjects, gingivitis and periodontitis patients was 0.15, 0.267 and 0.765 respectively (Fig. 3). A one way ANOVA analysis revealed that the absorber unit of periodontitis patients was significantly much higher than that of gingivitis patients and periodontal healthy subjects ($p<0.01$). Since absorbance unit represented the levels of these periodontopathic bacteria in the dental plaques, these results indicated that increased levels of dental plaque P. *gingivalis* were associated with increased severity of periodontal disease.

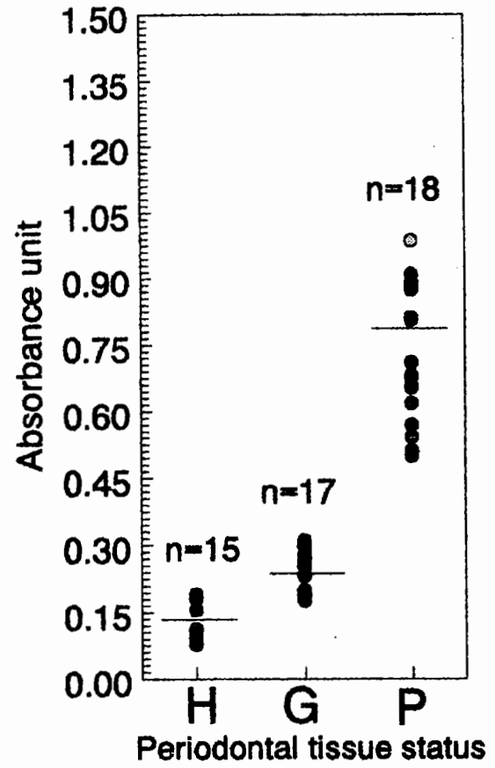

Figure 3. Levels of *P. gingivalis* in the dental plaques of periodontal diseased patients. Dental plaque samples of subjects were diluted in 1:10. Absorbance units represent the level of bacteria semiquantitatively. Dots represent an individual subject. Bars represent mean of each group. H = healthy, G = gingivitis, P = periodontitis.

Discussion

Since the discovery of a technique to propagate indefinitely monoclonal antibodies was reported, the use of such immunotechnique as a diagnostic tool in dentistry has been tremendous. Monoclonal antibodies specific to the whole proteins of *P. gingivalis* have been established and further characterizations revealed that these IgG2b antibodies did not cross react with other periodontopathic and non-oral bacteria (Simonson *et al.*, 1986). Unlike this previous report, the antibodies produced by clone Pg.C.5. are an IgG2a isotype. It seems plausible that distinct IgG subclasses obtained by the previous and present study may be related with different antigen preparations used. As yet, whether no cross reaction to other bacteria as seen by both studies is a common phenomenon of antibodies specific to *P. gingivalis* remains to be investigated further.

Studies have shown that increased levels of serum antibodies specific to LPS-Pg were observed in periodontal diseased patients as compared to the periodontal healthy control and these antigen-specific antibodies were predominantly an IgG2 isotype (Ogawa *et al.*, 1990; Lopatin and Blackburn, 1992; Choi *et al.*, 1996; Pietrzak *et al.*, 1998). Therefore, that clone Pg.C.5 produced IgG2a antibody isotype is not surprising and certainly supports the previous findings.

No cross reaction to LPS derived from other periodontopathic or non-related bacteria is of interest. Although biochemical structures of LPS from periodontopathic bacteria appear to be relatively similar, there are structurally several fundamental differences among periodontopathic bacteria-derived LPS. With respect to a lipid A composition, LPS isolated from *F. nucleatum* and A. *actinomycetemcomitans* are closely related with one derived from enteric bacteria, such as *E. coli*. On the other hand, lipid A of LPS-Pg does not have the 4'-O phosphorylated group of the glucosamine disaccharide, but it has relatively long acyl chain (Hamada *et al.*, 1990; Hofstad *et al.*, 1993). Consequently, toxic activity of LPS-Pg is much lower than that of enteric bacteria. It remains, therefore, to be seen as to whether no cross reactivity of LPS-Pg-specific monoclonal antibodies to LPS of other tested periodontopathic bacteria may be due to the fact that the antibodies only recognize a LPS-Pg-specific

epitope which is absent in other periodontopathic bacteria.

The use of monoclonal antibodies for the microbial detection as well as diagnosis of periodontal disease has been reported. For example, Wolff and colleagues (1992) have utilized fluorescence labeled anti-*P. gingivalis* monoclonal antibodies to detect dental plaque *P. gingivalis*. The results of this study showed that these antibodies were able to enumerate up to 10^4 bacteria. Even this immunotechnique was rapid and ease, high background might still occur. An ELISA-based bacterial enumeration by using these antigen-specifc antibodies has also been carried out and in fact, the results of this particular study were comparable, if not far better, to the one using the DNA probe (Melvin *et al.*, 1994). Indeed, the present study has also proven the usefulness of anti-*P. gingivalis* antibodies in detecting dental plaque *P. gingivalis of* periodontal diseased patients. It should however be kept in mind that dental plaque consists of mixed oral bacterial types, indicating that monitoring one particular bacterial type requires a specific and accurate method. A possibility that the results of the present study may also be contaminated by identification of periodontopathic bacteria other than *P. gingivalis* in such mixed bacterial dental plaques is unlikely to occur, since the antibodies used in this study specifically recognized the later bacteria.

Of interest, antigen-specific antibodies produced by clone Pg.C.5 were able to distinguish the levels of *P. gingivalis* of distinct periodontal disease stages. It is now known that the course of CIPD is highly correlated with different bacterial types colonized in the dental plaques and that P. *gingivalis* bacteria are predominantly isolated from dental plaque of patients with periodontitis but not periodontal healthy subjects or patients with gingivitis (Kamma *et al.*, 1994 and 1995; Darveau *et al.*, 1997). In this respect, increased levels of *P. gingivalis* of periodontitis patients as seen in the present study seem in accordance with such notions.

Whether an ELISA utilizing clone Pg.C.5-produced antigen-specific antibodies could be used to routinely monitor the presence of *P. gingivalis* as one of the diagnostic tools remains to be further investigated. The present study only semiquantitatively enumerated the levels of this bacterium, since the ability of these antibodies to precisely account the number of this bacterium as a positive control in each ELISA plate was not known. It is safely to say, however, that the ability of these antibodies to determine the levels of *P. gingivalis* as judged by the absorbance unit may be useful for epidemiological rather than for clinical studies, since the former one only requires a rapid and ease method to gain a general, not detail, periodontal health status of large populations. In summary, the present study has shown that LPS-Pg-specific IgG2a monoclonal antibodies were able to detect the presence of *P. gingivalis* in the dental plaque isolated from periodontal diseased patients. The levels of this periodontopathic bacterium in the dental plaque of patients with periodontitis were higher than those of patients with gingivitis and periodontal healthy subjects.

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