Original Article

Antibody Titer Against Porphyromonas Gingivalis in Rats with Experimentally Induced Periodontitis

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Abstract

Background and Aim: This study aimed to assess the antibody titer against Porphyromonas gingivalis (P. gingivalis) in rats with experimentally induced periodontitis.

Materials and Methods: Thirty-three Surrey rats were randomly divided into three groups (n=11). Group 1 was vaccinated with formalin-killed whole cell (FKWC) P. gingivalis with incomplete Freund's adjuvant as vaccine carrier and orally inoculated with viable P. gingivalis (ATCC 33277). Group 2 was vaccinated with incomplete Freund's adjuvant and PG buffer and orally inoculated with viable P. gingivalis (positive control). Group 3 was vaccinated as group 2 without inoculation (negative control). Two weeks later, they were vaccinated with a booster dose. One week later, serum and saliva samples were obtained to assess antibody titer. Oral inoculation of bacteria was then done four times every 48 hours. Two weeks later, serum, saliva and subgingival plaque samples were obtained from the maxillary second molar area for assessment of P. gingivalis count in the subgingival plaque. Data were analyzed using Mann-Whitney U test and Wilcoxon signed-rank tests.

Results: Serum and salivary antibody titers against P. gingivalis in group 1 one week after booster dose and two weeks after oral inoculation of bacteria were significantly different from those in other groups ($P<0.05$). Groups 1 and 3 were not significantly different in terms of bacterial count in subgingival plaque ($P=1.000$) but the difference between groups 1 and 2 was significant ($P<0.001$).

Conclusion: Vaccination with FKWC P. gingivalis increased serum IgG and salivary IgA and limited the colonization of P. gingivalis in subgingival plaque of rats.

Key Words: Immunization, Porphyromonas gingivalis, Periodontitis, Rats

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Introduction

Periodontal disease is the result of complex interactions of subgingival microbial biofilm and host immune and inflammatory responses, resulting in gingival and periodontal inflammation and eventual tooth loss. It mainly occurs due to the activity of Gram-negative microorganisms in the subgingival biofilm especially red complex microorganisms such as Porphyromonas gingivalis (P. gingivalis), Treponema denticola and Tannerella

forsythia [1-3].

Conventional periodontal therapy is non-specific and is mainly based on mechanical plaque and calculus removal and periodontal surgery [1]. This modality is costly and unfavorable for patients and has a variable prognosis [3]. At present, P. gingivalis is known as the main periopathogenic microorganism responsible for occurrence of chronic periodontitis [4-6]. It is a rod-shaped, asaccaharolytic, non-mobile, anaerobic Gram-negative microorganism from the family of black pigmented microorganisms, which can stimulate the humoral immune response via its several virulence factors including lipopolysaccharides, cysteine proteases known as gingipains, fimbria, extracellular DNA and severe invasion to tissues [1-3, 7].

In mammals, vaccination with killed whole cells of P. gingivalis significantly decreases the progression of periodontitis caused by the oral microflora such as infection with P. gingivalis [3]. Moreover, in vaccination with whole cells of P. gingivalis, reduction in bone loss has been reported in rats with periodontitis [3-6, 8]. Sharma et al. [8] used fimbria of P. gingivalis for vaccination of rats and noticed relative immunization (60%) against periodontitis induced by P. gingivalis.

Wilensky et al. [9] showed that immunization with recombinant RgpA peptide prevented alveolar bone loss similar to heat-killed whole cell P. gingivalis bacteria.

Findings of animal studies support the use of P. gingivalis vaccine as an adjunct for treatment of chronic periodontitis in humans. Accordingly, we selected P. gingivalis since it can stimulate the humoral immune response in hosts via several virulence factors such as outer membrane vesicles containing virulence factors such as LPS and gingipains, arginine, lysine cysteine proteases and fimbria [3,4,10]. We used the formalin killed whole cell (FKWC) P. gingivalis for vaccination of rats to assess its role in achieving acquired active immunity as a preliminary step for use of such vaccines for human periodontitis.

Materials and Methods

Obtaining P. gingivalis:

P. gingivalis (ATCC33277) was obtained from SinaClone company (Figure 1). Based on a previous study and the company's instructions [3], bacteria were stored at room temperature in lyophilized cultures. Cultured bacteria were transferred to sheep blood agar plate containing 10v% lysed sheep blood, 15 µg/mL hemin and 1 µg/mL menadione and stored in gas pack A anaerobic jar at 37°C. Three to five-day culture of P. gingivalis (ATCC33277) was transferred to brain heart infusion broth enriched with 15 µg/mL hemin, 1 µg/mL menadione, cysteine (1 µL/mL) and 0.5 g/L chloric acid at 37°C [3,6]. Pure culture was Gram-stained and the morphology of colonies was evaluated, which indicated Gram-negative coccobacilli. The brain heart infusion broth containing bacteria was centrifuged at 10,000 g for 30 minutes at 4°C and the supernatant was removed. The cell sediment was then reached to the final concentration of $2.5x10^{11}$ colony forming units (CFUs)/mL by adding PG buffer (150mM NaCl, pH of 7.8, 50mMTris-Hcl, 0.5 g/L cysteine, 10mM MgSO4) at 4°C containing 5% carboxymethyl cellulose with low viscosity for inoculation into the oral cavity of rats [3,6].

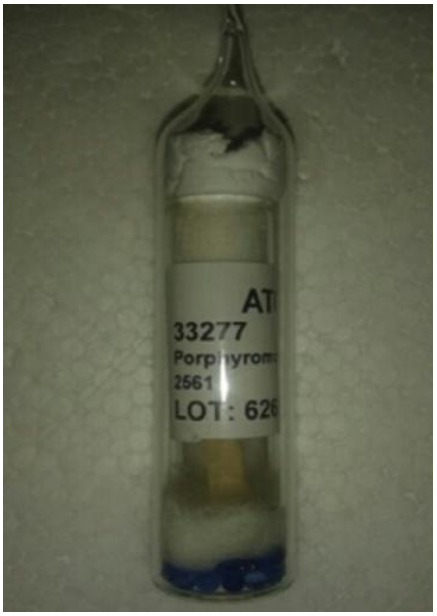

Figure 1. Lyophilized P. gingivalis (ATCC33277)

Preparation of FKWC P. gingivalis (ATCC33277):

Bacteria in equal volume with 0.5% saline were incubated overnight in a shaking incubator. Sterile PG buffer (10 times the volume of cells) was

added to the cells and the suspension was centrifuged at 10,000 g for 10 minutes. The supernatant was removed and the cell sediments were gently immersed in PG buffer again (20 times the cell sediment volume) and centrifuged for another 10 minutes. After removing the supernatant, cells were immersed again in sterile PG buffer to obtain 10^{10} CFUs per 0.1 mL of PG buffer containing 5% carboxymethyl cellulose. For immunization, cell suspension was mixed with incomplete Freund's adjuvant (IFA) in 1:1 ratio and injected to rats as follows [3,6].

After obtaining ethical approval from the ethics committee of our university, 33 male Surrey rats free from black pigmented pathogens were randomly divided into three groups of 11 and housed in cages. We made sure that the rats were free from black pigmented pathogens such as P. gingivalis. Plaque samples were obtained of all rats and cultured in sheep blood agar plate supplemented with 400 µg/mL kanamycin.

Group 1: Rats in group 1 were vaccinated with FKWC P. gingivalis (10^{10}) and ICFA in 1:1 ratio and were also inoculated with P. gingivalis. Group 2: Rats in group 2 were vaccinated with ICFA+PG buffer and their oral cavity was inoculated with P. gingivalis (positive control).

Group 3: Rats in group 3 were vaccinated with ICFA+PG buffer but were not inoculated with P. gingivalis (negative control).

Four-week-old rats in groups 1-3 were vaccinated with 0.2 mL of the suspension via a subcutaneous injection at the back of their neck. All groups were vaccinated again with the same dose two weeks after the first vaccination (booster) [3, 4, 6]. One week after booster injection, rats were anesthetized with ether and blood was drawn from their retrobulbar vascular network using a capillary tube. After centrifugation, serum was stored at-70°C. Saliva samples were also obtained using a 1 mL sampler and after centrifugation, they were also stored at -70°C.

Fifteen days after vaccination, all animals received antibiotics via intraperitoneal injection for three consecutive days to suppress endogenous microflora. Each rat received 20 mg ampicillin and kanamycin daily with 0.5 mL of 5% carboxymethyl cellulose solution in water.

At 21 days after sampling, all animals in groups 1 and 2 were inoculated with P. gingivalis (ATCC33277). This was repeated four times with 48-hour intervals. The rats were inoculated with viable P. gingivalis by a 1 mL insulin syringe fitted to a 16-gage gavage tube. Each rat was inoculated with 1 mL of P. gingivalis suspension as follows: 0.75 mL of the suspension was gavaged; 0.2 mL of the suspension was inoculated to the molar tooth gingiva (50 µL per each quadrant) and 50 µL was inoculated to the colorectal area.

Two weeks after the final inoculation of viable bacteria, saliva and serum samples were obtained again to assess antibody titers. Samples were also taken from the subgingival plaque at the maxillary second molar site to assess the number of bacteria in the subgingival plaque.

To collect the saliva, pilocarpine nitrate (5 mg/kg) was injected to the back of their neck for parasympathetic stimulation. Saliva was collected by a 1 mL sampler. Collected saliva was stored in 1.5 mL micro-centrifuge tubes on ice until centrifugation. After centrifugation at 5000 g for 20 minutes, the supernatant was frozen at -70°C for further analyses.

Antibody in serum and saliva samples was analyzed using ELISA kit. To assess serum and saliva total antibody titer, ELISA Ensemble kit (Alpha Diagnostic, San Antonio, Texas, USA) specific for rats (#80155) was used. To determine the class of antibody, IgG FC and IgA FC HRP conjugated goat anti-rat (KOMA Biotech) kit was used.

For subgingival plaque sampling, a thin paper point was used. The collected sample was transferred to sodium thioglycolate culture medium containing 15 µg/mL of hemin and 1 µg/mL of menadione. They were transferred to lab within 30 minutes for culture in sheep blood agar containing 400 µg/mL kanamycin. Culture plates were placed in gas pack A anaerobic jar. After five days, the plates were evaluated for presence of P. gingivalis. First, each colony was Gram-stained. P. gingivalis colonies are shiny, convex, dark brown to black in color, have a bad odor, form a mucoid plaque and are without florescence. They are in the form of bacilli and coccobacilli. Presence of P. gingivalis was also confirmed by biochemical tests. Catalase test was negative and indole test was positive. Bacteria were sensitive to vancomycin disc

(presence of growth inhibition zone) and resistant to kanamycin and colchicine. Urease test, bile esculin hydrolysis and glucose, lactose and sucrose fermentation were all negative.

The characteristics of selected samples were described using central and dispersion indices. The Mann-Whitney U test and Wilcoxon signed-rank test were used to compare the two groups and for within group comparisons, respectively. $P<0.05$ was considered significant. Statistical calculation and data management were performed using SPSS 21 (SPSS Inc., IL, USA).

Results

The Mann-Whitney U test was used to analyze the data. The serum and salivary antibody titers (IgG and IgA) at one week after booster injection and two weeks after final inoculation in group one were significantly different from the antibody titers in the other two groups ($P<0.05$). At one week after booster injection, antibody titer in groups 2 and 3 was undetectable, which indicated that FKWC could induce immunization in group 1. But at two weeks after oral inoculation of bacteria, antibody level was slightly detectable in group 2, which was probably due to cross-reaction of immune system with viable bacteria (Table 1 and Diagram 1).

As could be seen in Table 2, number of bacteria in subgingival plaque in group 1 was similar to that in group 3 and it was undetectable (negative control) but Wilcoxon Signed-Rank test showed a significant difference with the positive control group (2.2×10^3) ($P=0.000$), which indicated the ability of FKWC to prevent bacterial colonization (Table 2 and Diagram 2).

Table 1. IgG and IgA antibody titers one week after booster and two weeks after inoculation

Group		IgG at 1 week after booster	IgG 2 weeks after inoculation	IgA at 1 week after booster	IgA 2 weeks after inoculation
FKWC P. gingivalis IgG + Inoculation	N	11	11	11	11
	Mean	17363.64	41909.09	1212.73	1237.27
	Std. Deviation	27540.053	46131.236	2947.077	2936.338
IFC buffer PG (no inoculation)	N	11	11	11	11
	Mean	1.00	1.00	1.00	1.00
	Std. Deviation	.000	.000	.000	.000
IFC buffer PG + inoculation	N	11	11	11	11
	Mean	1.00	23.09	4.27	6.73
	Std. Deviation	.000	38.261	4.541	4.541
P value	<0.001	<0.001	<0.001	<0.001	<0.001

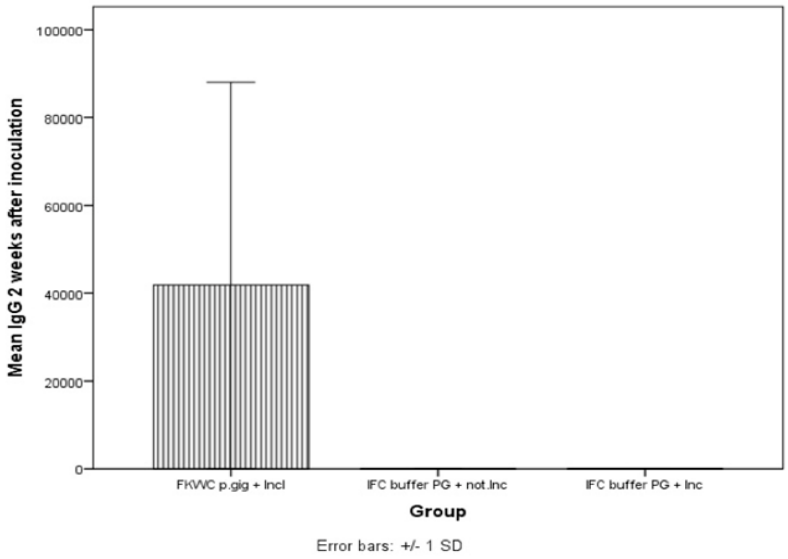

Diagram 1. Antibody level at two weeks after oral inoculation of bacteria

Table 2. Bacterial count at two weeks after inoculation

Group		Bacterial Count 2 weeks after last inoculation	P value
FKWC P. gingivalis IgG + inoculation	N	11	P<0.001
	Mean	0.00	
	Std. Deviation	0.000	
IFC buffer PG (no inoculation)	N	11	
	Mean	0.00	
	Median	0.00	
	Std. Deviation	0.000	
IFC buffer PG + inoculation	N	11	
	Mean	2200.00	
	Std. Deviation	635.610	

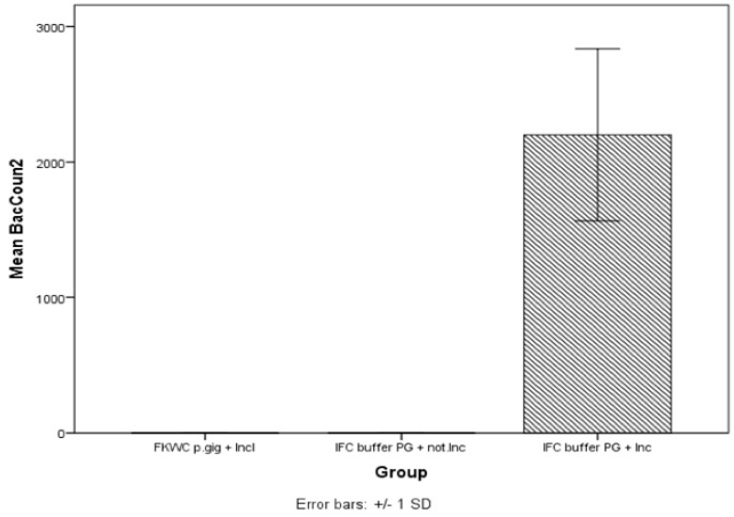

Diagram 2. Mean bacterial count in the three groups

Discussion

The results of our study showed that vaccination of rats with FKWC P. gingivalis (ATCC33277) increased the titers of IgG and IgA antibodies and prevented bacterial colonization in subgingival plaque after inoculation of rats. In rats vaccinated with IFA and PG buffer, no increase in titer of specific antibody against P. gingivalis was noted. This finding implied that the carrier of vaccine (IFA and PG buffer) was not able to stimulate the immune system to produce antibody.

Sampling from the subgingival plaque showed absence of P. gingivalis in plaque samples of rats vaccinated with FKWC and the negative control group that did not receive bacterial inoculation compared to groups vaccinated with IFA and PG buffer and inoculated with P. gingivalis. These results indicated that FKWC vaccination prevented colonization of P. gingivalis in subgingival plaque and that this finding indicates that it may prevent or reduce the occurrence of periodontitis in vaccinated rats. In rats vaccinated with IFA and PG buffer and inoculated with P. gingivalis, high levels of P. gingivalis were isolated from the subgingival plaque. This finding implies that the carrier of vaccine (IFA and PG buffer) was not able to protect the animals against P. gingivalis. Rats immunized with FKWC showed high titers of serum IgG; these results were in line with the findings of Rajapaks et al, [6] and Genco et al. [11] used competitive ELISA and showed production of 42% and 53% antibody against FKWC and RgpA-Kgp following vaccination of rats.

Wilensky et al. [9] showed that immunization with recombinant RgpA peptide prevented alveolar bone loss similar to heat-killed whole cell P. gingivalis bacteria and stimulated immune response towards anti-inflammatory response by reduction in IL-1B pro-inflammatory cytokine and increase in IL-4.

Puth et al, [12] also showed that mucosal immunization with a flagellin-adjuvanted Hgp44 induced a significantly higher Hgp44 specific IgG titer in the sera of mice and significantly decreased alveolar bone loss. Yonezawa et al. [13] showed that DNA vaccine contained RgpA gene, induced antibody against kgP39, RgP27 and RgpA44 in P. gingivalis and prevented the hemagglutination of bacteria and bond to type I collagen. In our study, specific IgA antibody was isolated from the saliva against FKWC in rats vaccinated with FKWC but in group vaccinated with IFA and PG buffer and inoculated with P. gingivalis, a low level of antibody was noted.

These results may indicate that vaccination stimulates B cells to produce IgA and in presence of P. gingivalis, mucosal lymphoid tissue can secrete Th2 cytokines for maturation of B cells,

which secrete IgA in the saliva [1-3]. The main antibody induced by FKWC vaccination in serum is IgG, which highlights significant activity of Th2 cells. IgG antibody in rats induces phagocytosis and antibody-dependent cytotoxicity by macrophages and natural killer cells through FCγRII receptor [1-3]. Moreover, IgG prevents degranulation of mast cells during phagocytosis and endocytosis [3]. IgG2a-specific antibody against RgP-kgp epitopes is present in gingival tissue and gingival crevicular fluid. Thus, antibodies may also be present in the saliva. In vaccinated rats, IgG-specific antibody is secreted into the saliva. Presence of specific antibody in the saliva and gingival crevicular fluid may prevent attachment and colonization of P. gingivalis in the oral cavity, which explains undetectable bacteria in plaque samples of rats vaccinated with FKWC [1,3,5].

Thus, presence of IgG2 antibody in the gingival tissue may prevent penetration of RgpA-kgp (a major virulence factor) into the tissue. Opsonization of RgpA-kgp, extra-membranous vesicles or invading cells by IgG2 antibody induces phagocytosis mediated by FC receptor and slight expression of pro-inflammatory cytokines [1-3,5,6]. These protective mechanisms may explain why FKWC vaccination prevented periodontitis induced in rats by oral inoculation of P. gingivalis. Sharma et al. [8] used fimbria of P. gingivalis for vaccination of rats and noticed relative immunization (60%) against periodontitis induced by P. gingivalis. They concluded that Fim A may not be the main virulence factor of P. gingivalis. Another study evaluated sera of 25 patients with chronic periodontitis and 25 healthy controls to determine IgG antibody (and its subgroups) response to RgpA-kgp [3]. In sampling from subgingival plaque of patients, a correlation was noted between the severity of disease and percentage of sites containing P. gingivalis. The patient group showed high IgG specific response to RgpA-kgp compared to the control group, and response rate was significantly correlated with the mean probing depth and percentage of sites positive for P. gingivalis. Thus, vaccination wit FKWC or RgpA-kgp can be considered as an adjunct for prevention of chronic periodontitis.

Conclusion

Our results showed that vaccination of rats with FKWC P. gingivalis increased the serum IgG and salivary IgA antibody titers and limited the colonization of P. gingivalis in the subgingival plaque. These findings indicate that this vaccination may be efficient for prevention of periodontal disease in humans as well.

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