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Molecular mimicry of *Aggregatibacter actinomycetemcomitans* with β2 glycoprotein I

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Introduction: β2-Glycoprotein I (β2GPI) is important in the suppression of coagulation, and antibodies against TLRVYK peptides on the β2GPI molecule are related to thrombosis. According to the Swiss-Prot database, *Aggregatibacter actinomycetemcomitans* leukotoxin c has sequences (SIRVYK) that are homologous to the TLRVYK peptides. The aim of this study was to investigate the effects of *A. actinomycetemcomitans* infection on the antibody response against SIRVYK peptides in patients with periodontitis.

Methods: Serum immunoglobulin G (IgG) antibody and IgG subclass antibody titers against SIRVYK or TLRVYK peptides were measured by enzyme-linked immunosorbent assay in 46 patients with aggressive periodontitis (eight with localized disease, 38 with generalized disease), 28 patients with chronic periodontitis, and 20 periodontally healthy subjects. The presence of *A. actinomycetemcomitans* in plaque and saliva samples was determined using polymerase chain reaction.

Results: The level of anti-SIRVYK antibodies was significantly higher in patients who were *A. actinomycetemcomitans*-positive than in *A. actinomycetemcomitans*-negative patients ($P < 0.05$) in the chronic periodontitis group. A similar trend was found in the antibody response to TLRVYK peptide; however, no statistically significant difference was seen between *A. actinomycetemcomitans*-positive and -negative patients. The *A. actinomycetemcomitans*-positive patients displayed significantly higher levels of anti-SIRVYK IgG2 and IgG3 antibodies than *A. actinomycetemcomitans*-negative patients ($P < 0.05$ and $P < 0.05$, respectively). The level of IgG2 was highest among the four IgG subclasses and it predominantly increased in patients who were *A. actinomycetemcomitans*-positive. Anti-TLRVYK antibody levels were significantly correlated with anti-SIRVYK IgG antibody levels.

Conclusion: The results suggest that *A. actinomycetemcomitans* infection may elicit anti-SIRVYK IgG antibodies and modify the anti-TLRVYK antibody response in patients with periodontitis by molecular mimicry with β2GPI.

Key words: *Aggregatibacter actinomycetemcomitans*; β2 glycoprotein I; molecular mimicry

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Antiphospholipid syndrome is an autoimmune disorder associated with arterial and venous thrombosis and recurrent fetal loss. It is characterized by the presence of antiphospholipid antibody including anticardiolipin antibody and lupus anticoagulant. An initial report suggested that the specificity of the antiphospholipid antibody was directed to anionic phospholipids (10). Later, it was reported that β2-glycoprotein I (β2GPI), which binds to exposed phospholipids, was the antigenic determinant for these antiphospholipid antibodies (9, 13, 24). The β2GPI is a plasma protein and acts as an anticoagulant by

inhibiting prothrombinase activity (14), adenosine diphosphate-induced platelet aggregation (15), and platelet factor IX production (20). Antiphospholipid antibodies inhibit the anticoagulant functions of β2GPI, and it has recently been shown that they directly activate endothelial cells to up-regulate adhesion molecules (17).

Schenkein et al. reported that the prevalence of β2GPI-dependent anticardiolipin autoantibodies was greater in patients with chronic periodontitis and generalized aggressive periodontitis (16.2 and 19.3%, respectively) than in healthy controls and in patients with localized aggressive periodontitis (6.8 and 3.2%, respectively) (19). Patients with these autoantibodies demonstrated increased pocket depth and attachment loss compared with patients lacking the antibodies (19).

Blank et al. suggested that viral or bacterial infections might be responsible for the generation of anti-β2GPI antibodies by molecular mimicry (4). Three hexapeptides were identified and were specifically recognized by pathogenic anti-β2GPI monoclonal antibodies, which caused endothelial cell activation and induced experimental antiphospholipid syndrome (6). Of these, TLRVYK peptides correspond to the third domain of β2GPI, and the peptidic domains of many pathogens, including *Salmonella typhi*, *Chlamydia*, *Helicobacter pylori*, and *Porphyromonas gingivalis*, have strong homology with the TLRVYK hexapeptide (5). According to the Swiss-Prot database, there is strong homology between TLRVYK in β2GPI and the peptidic domain (SIRVYK) in *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* leukotoxin c. No studies have reported on the molecular mimicry between β2GPI and periodontal pathogens. This study aimed to investigate the effects of *A. actinomycetemcomitans* infection on the antibody response against SIRVYK peptide, a homologue of TLRVYK peptide, in patients with periodontitis.

Materials and methods

Study population

Patients with periodontitis attending the Periodontics Clinic at the Tokyo Medical & Dental University were selected for this study: eight had localized aggressive periodontitis, 38 had generalized aggressive periodontitis, and 28 had generalized chronic periodontitis. Twenty periodontally healthy subjects served as controls. Patients with periodontitis were diagnosed according to the classification of the 1999 American Academy of Periodontology workshop (8, 23); the detailed criteria have been described previously (25).

Informed consent was obtained from each subject. All subjects were evaluated by measuring clinical parameters such as probing depth, bleeding on probing, and oral radiographs. Healthy subjects showed no clinical or radiographic evidence of periodontal disease.

All subjects were generally healthy and had not received any periodontal treatment during the previous 6 months or systemic antibiotic administration during the previous 3 months.

Bacterial sampling and detection by polymerase chain reaction

Subgingival plaque samples were collected with a paper point from the deepest pockets in each quadrant in the patients with periodontitis, and from the mesiobuccal surface of #16, #21, #36, and #41 in healthy subjects. Approximately 1 ml of unstimulated saliva was also collected from each individual in a sterile plastic tube.

Subgingival plaque and whole saliva samples were subjected to a 16S ribosomal RNA-based polymerase chain reaction detection method (3) to determine the presence of *A. actinomycetemcomitans*. The plaque samples were analyzed as individual site samples, and subjects were designated as positive for *A. actinomycetemcomitans* if the subject had at least one positive sample in subgingival plaque and/or saliva samples.

Serum samples

Peripheral blood samples were collected from the patients and healthy subjects. Each blood sample was centrifuged at 1500 ***g*** for 20 min. The serum was filtered through a sterile 0.45-mm diameter filter (Millex; Millipore Japan Ltd., Tokyo, Japan) and stored at −20°C until analysis.

Enzyme-linked immunosorbent assay to detect antipeptide antibody

The rabbits were immunized with keyhole limpet hemocyanin-conjugated TLRVYK peptide with Freund's complete adjuvant once a week for 4 weeks, and immune sera were then collected. Immune rabbit serum showed antipeptide reactivity in an enzyme-linked immunosorbent assay (ELISA) whereas preimmune serum from the same rabbit did not (data not shown). Anti-TLRVYK rabbit immunoglobulin was purified by affinity chromatography with a sepharose column conjugated with TLRVYK peptide.

Antibody responses to TLRVYK peptides were determined by ELISA. Briefly, 96-well assay plates (Costar, Cambridge, MA) were incubated with TLRVYK peptide (10 μg/ml) and blocked with 3% bovine serum albumin. As a standard, two-fold serial dilution (50 ng/ml to 0.098 ng/ml) of rabbit anti-TLRVYK purified immunoglobulin G (IgG) was added to the top two rows of the plate. The remaining rows were prepared with diluted (1 : 128) sera from patients, in duplicate, and the plates were incubated overnight at 4°C. As protein A binds to both human and rabbit IgG, the binding was detected using protein A-peroxidase (1 : 1000), followed by use of the tetramethylbenzidine liquid substrate system (Sigma, St Louis, MO). The reaction was stopped with 1 M H_2SO_4 (Sigma) after 30 min and read at 450 nm using a microplate reader (Soft Max™). Extensive washing with phosphate-buffered saline containing 0.05% Tween-20 (PBS-T) followed each step. Data were analysed by comparison with standard curves, and titer concentrations are expressed as immunoglobulin in μg/ml.

Specific IgG-subclass antibodies to SIRVYK peptide

Levels of serum IgG subclass antibodies to synthetic peptide were determined using a modification of previously described methods (2, 21). The top two rows of each 96-well assay plate (Costar) were coated with 0.1 ml of serial two-fold dilutions (100 ng/ml to 0.196 ng/ml) of the purified monoclonal antibodies (human IgG1, IgG2, IgG3 or IgG4 kappa and lambda; Sigma), which were used as the standard. The remaining rows were coated with 0.1 ml of 10 μg/ml SIRVYK peptide in carbonate buffer, and incubated overnight at 4°C. After blocking with 200 μl 2% bovine serum albumin, plates were washed three times with PBS-T. A single dilution of serum (1 : 100), which was determined from the linear part of each serum dilution curve in preliminary experiments, was added to antigen-coated wells in duplicate, and the plate was incubated for 2 h at 37°C. Following incubation and washing, the secondary antibody consisted of 100 μl of each concentration of monoclonal anti-human biotin conjugate for IgG1 (8c/6-39; Sigma), IgG2 (HP6014; Sigma), IgG3 (HP6050; Sigma), and IgG4 (HP6025; Sigma). Plates were incubated for 2 h at

37°C, and then washed three times with PBS-T. Subsequently, streptavidin–peroxidase (1 : 1000) was added and incubation was performed for 1 h at 37°C. After washing, the plates were developed using the tetramethylbenzidine liquid substrate system (Sigma) and the reaction was stopped with 1 M H_2SO_4 (Sigma) after 30 min. Data were analyzed by comparison with standard curves, and titres are expressed as immunoglobulin in μg/ml (21).

Statistical analysis

The data were analyzed with the subject as the unit and were reported as median and interquartile range. All statistical comparisons among the four groups were performed with Kruskal–Wallis analysis of variance. The Mann–Whitney *U*-test was used to estimate the significance of differences between patients with periodontitis and healthy subjects, and between *A. actinomycetemcomitans*-positive and -negative subjects. Correlation between anti-TLRVYK antibodies and anti-SIRVYK antibodies was evaluated using Spearman's rank correlation test. The test was two-tailed, and $P < 0.05$ was considered statistically significant.

Results

Anti-TLRVYK and anti-SIRVYK antibodies in patients with periodontitis and healthy subjects

The levels of IgG to peptides (TLRVYK and SIRVYK) in patients with various forms of periodontitis and healthy subjects are shown in Table 1. No significant difference was found in anti-TLRVYK antibody among the four groups. On the other hand, anti-SIRVYK antibodies were significantly elevated in the chronic periodontitis and generalized aggressive periodontitis groups compared with healthy subjects ($P < 0.05$ and $P < 0.05$, respectively).

Anti-TLRVYK and anti-SIRVYK antibodies in *A. actinomycetemcomitans*-positive and -negative subjects

A. actinomycetemcomitans was detected in patients with chronic periodontitis (25%) and with generalized aggressive periodontitis (24%), but was not found in patients with localized aggressive periodontitis (0%) or in healthy subjects (0%) (Table 2). Levels of antibodies to TLRVYK and SIRVYK peptides in *A. actinomycetemcomitans*-positive and -negative subjects are summarized in Fig. 1. In the group of patients with chronic periodontitis, the level of anti-SIRVYK antibodies was significantly higher in *A. actinomycetemcomitans*-positive patients than in *A. actinomycetemcomitans*-negative patients ($P < 0.05$). A similar trend was found in the group with generalized aggressive periodontitis, although the difference was not significant. The level of anti-TLRVYK antibodies was also higher in *A. actinomycetemcomitans*-positive patients than in *A. actinomycetemcomitans*-negative patients in the group with chronic periodontitis, but the difference failed to reach statistical significance.

Table 1. Specific immunoglobulin G antibodies to SIRVYK peptide in patients with periodontitis and healthy subjects

Subjects	Antibodies to peptides (μg/ml)[1]	
	TLRVYK	SIRVYK
Chronic periodontitis (n = 28)	0.34 (0.23, 0.60)	2.30 (1.47, 3.09)*
Generalized aggressive periodontitis (n = 38)	0.31 (0.25, 0.51)	2.23 (1.48, 3.03)*
Localized aggressive periodontitis(n = 8)	0.34 (0.30, 0.62)	2.46 (0, 3.16)
Healthy (n = 20)	0.35 (0.24, 0.48)	1.09 (0, 1.97)

[1]Median (interquartile range).
*Significantly higher than those in healthy subjects.

Table 2. Clinical parameters and microbiological data

	Chronic periodontitis	Aggressive periodontitis		
		Generalized	Localized	Healthy
Number of subjects	28	38	8	20
Age (years)[1]	54.9 ± 6.8	27.0 ± 5.8	22.8 ± 5.6	26.3 ± 3
Probing depth >4 mm (%)[1]	43.2 ± 26.1	73.0 ± 21.7*	21.9 ± 11.5	0
Bleeding on probing (% of sites)[1]	21.7 ± 22.7	38.9 ± 27.1	5.9 ± 3.5	0
A. actinomycetemcomitans detection rate (%)	25	24	0	0

[1]Mean ± SD.
*Significantly higher than those in chronic periodontitis and localized aggressive periodontitis.

Correlation between anti-TLRVYK and anti-SIRVYK antibodies

Figure 2 illustrates the relationship between anti-SIRVYK and anti-TLRVYK antibodies. A significant correlation between the two antibody responses was found in all subjects ($r = 0.269$, $P = 0.009$).

Anti-SIRVYK IgG subclass antibody in patients with periodontitis and healthy subjects

Patients with chronic or with generalized aggressive periodontitis had significantly higher levels of serum IgG1, IgG2, IgG3, and IgG4 antibodies against SIRVYK peptides compared with healthy subjects ($P < 0.01$) (Fig. 3). The median anti-SIRVYK peptide antibody level was highest for IgG2, followed by IgG4, IgG1, and IgG3 (Fig. 3).

Specific IgG subclass antibodies to SIRVYK peptide in *A. actinomycetemcomitans*-positive and -negative periodontitis patients

A. actinomycetemcomitans-positive patients had significantly higher levels of

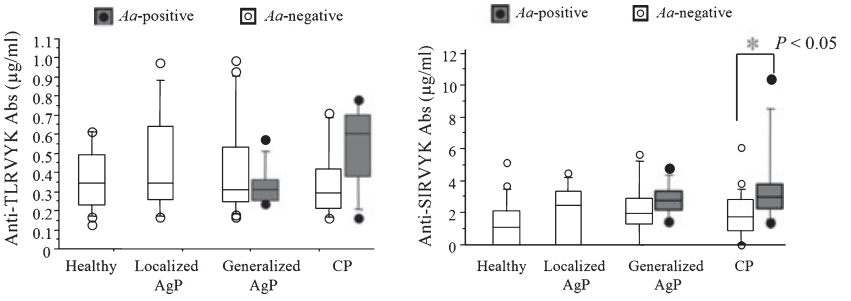

Fig. 1. Relationship between the presence of *Aggregatibacter actinomycetemcomitans* (*Aa*) and anti-TLRVYK and anti-SIRVYK antibodies in each group. The box represents the 25th (bottom) and 75th (top) percentiles and the median is the horizontal line inside the box. Black circles represent outliers; CP, chronic periodontitis; AgP, aggressive periodontitis.

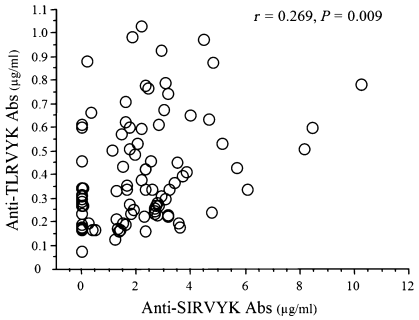

Fig. 2. Correlation between levels of anti-SIRVYK immunoglobulin G (IgG) antibody and anti-TLRVYK antibody in all subjects. The correlation coefficient (*r*) represents the relationship between anti-SIRVYK and anti-TLRVYK antibodies.

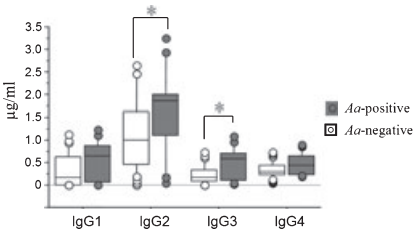

Fig. 4. Levels of immunoglobulin G (IgG) subclass antibodies to SIRVYK peptide in *Aggregatibacter actinomycetemcomitans*-positive and -negative periodontitis patients. $*P < 0.05$, significantly higher in *A. actinomycetemcomitans*-positive patients than in *A. actinomycetemcomitans*-negative patients.

anti-SIRVYK IgG2 and IgG3 antibodies than *A. actinomycetemcomitans*-negative patients (both $P < 0.05$) (Fig. 4). Elevated levels of anti-SIRVYK IgG1 and IgG4 were also found in *A. actinomycetemcomitans*-positive patients. However, the difference between *A. actinomycetemcomitans*-positive and *A. actinomycetemcomitans*-negative patients was not statistically significant.

Discussion

Molecular mimicry between bacterial and human antigens is involved in various autoimmune and infectious diseases. Schenkein et al. (19) reported an increased frequency of β2GPI-dependent anticardiolipin in both patients with chronic periodontitis and patients with aggressive periodontitis compared with healthy subjects. Data analysis indicates an increased level of anti-SIRVYK antibody in patients with chronic periodontitis and generalized aggressive periodontitis compared with healthy subjects. Furthermore, increased levels of anti-TLRVYK and anti-SIRVYK antibodies were seen in *A. actinomycetemcomitans*-positive patients with chronic periodontitis, which suggests that *A. actinomycetemcomitans* infection induced an antibody response to SIRVYK and even TLRVYK peptide. The level of anti-TLRVYK antibodies significantly correlated with the level of anti-SIRVYK antibodies in patients with periodontitis, suggesting molecular mimicry between β2GPI and *A. actinomycetemcomitans*. However, *A. actinomycetemcomitans* was not detected in plaque and saliva samples taken from patients with localized aggressive periodontitis (25). The increase in the level of anti-SIRVYK antibodies in *A. actinomycetemcomitans*-positive subjects was found in patients with chronic periodontitis but not in those with generalized aggressive periodontitis. In addition, the mean age of the chronic periodontitis group (54.9 ± 6.8) was significantly higher than that of the generalized aggressive periodontitis group (27.0 ± 5.8) in the present study. This indicates that a prolonged exposure to *A. actinomycetemcomitans* may be responsible for the clonal expansion of B lymphocytes specific to SIRVYK or TLRVYK peptide. These SIRVYK-specific B cells may gradually accumulate in patients with periodontitis, and subsequently patients with large numbers of these SIRVYK-specific B cells may produce large amounts of anti-SIRVYK antibodies in response to *A. actinomycetemcomitans*.

Several bacterial infections have been associated with increased levels of anti-β2GPI antibodies or antiphospholipid syndrome, and these bacteria share homologous peptides with β2GPI. *H. pylori* infection is an independent risk factor for cerebral ischemia (11) and there are some homologies between β2GPI and *H. pylori* (5). In addition, the disappearance of antiphospholipid antibodies after eradication of *H. pylori* has been reported (7). The presence of anti-*Saccharomyces cerevisiae* antibody is a main marker for Crohn's disease, and *S. cerevisiae* also has a homologous sequence with TLRVYK in its vacuolar protein sorting-associated protein (5). Episodes of thrombosis in Crohn's disease were accompanied with elevated titers of anti-β2GPI antibodies (1, 12, 22). In a large-scale case–control study, subjects who were IgA-seropositive for *A. actinomycetemcomitans* had a significantly higher odds ratio for the development of stroke compared with other subjects (16). As *A. actinomycetemcomitans* also has a peptidic domain (SIRVYK) with high homology to TLRVYK in β2GPI, long-term infection with *A. actinomycetemcomitans* may augment thrombogenic antibodies against β2GPI antibody by molecular mimicry.

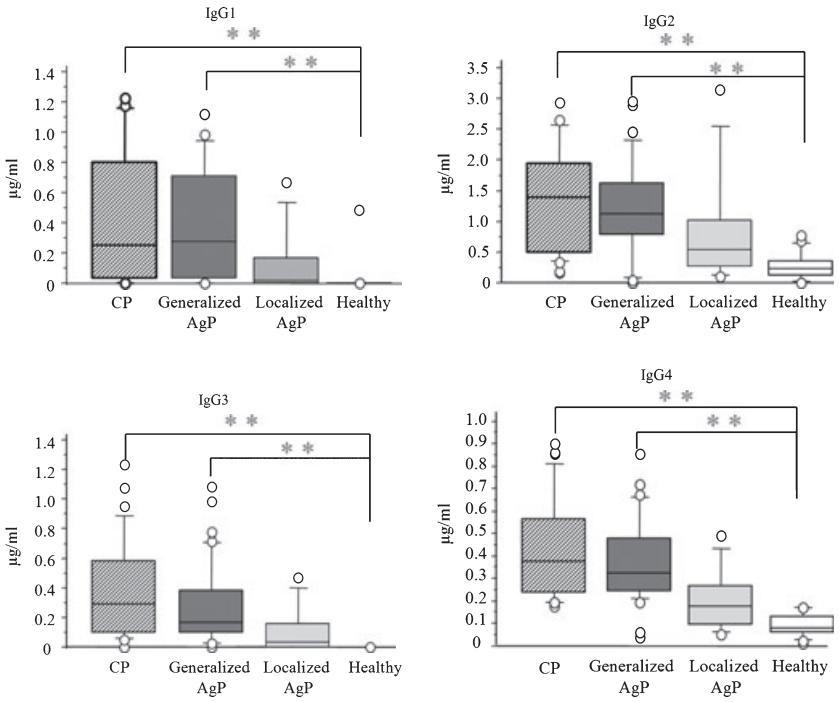

Fig. 3. Anti-SIRVYK immunoglobulin G (IgG) subclass antibodies in the four groups. $**P < 0.01$, significantly higher in the chronic periodontitis (CP) and generalized aggressive periodontitis (AgP) groups than in healthy subjects.

The anti-SIRVYK IgG2 antibody response was highest among the IgG subclass antibodies. Samarkos et al. examined the IgG subclass distribution of anticardiolipin and anti-β2GPI antibodies in patients with lupus erythematosus and in patients with primary antiphospholipid syndrome. They found a predominance of IgG2 among anti-β2GPI antibodies, and the antibody was associated with thrombosis (18). As IgG2 antibody responses are generally directed to carbohydrate antigens, TLRVYK and SIRVYK peptides may both be recognized in the context of carbohydrate antigens.

Besides *A. actinomycetemcomitans*, *P. gingivalis* also has a sequence (TLRIYT) that is highly homologous with TLRVYK peptide (5). *P. gingivalis*, a major periodontal pathogen, was detected at a high level in the plaque and saliva taken from patients with periodontitis in our study population (data not shown). It may be a possible confounding factor, and the effect of *P. gingivalis* infection on the immune response against TLRVYK peptide is currently under investigation.

In conclusion, the results of this study suggest that *A. actinomycetemcomitans* infection may elicit anti-SIRVYK IgG antibodies and modify the anti-TLRVYK antibody response in patients with periodontitis by molecular mimicry with β2GPI.

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