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Salivary immunoglobulin A directed to oral microbial GroEL in patients with periodontitis and their potential protective role

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The aim of this study was to identify salivary immunoglobulin A (IgA) directed to oral microbial GroEL in patients with periodontitis and to demonstrate their potential protective role through a reduction of inflammatory cytokine production induced by microbial GroEL. Using five different proteins belonging to the heat-shock protein 60 family, Western immunoblot analysis of salivary IgA from 63 subjects revealed immunoreactivities with *Campylobacter rectus* GroEL and *Porphyromonas gingivalis* GroEL in subjects with periodontitis ($P < 0.05$) compared to control subjects. Using the BIACORE 1000 to measure the salivary IgA titers directed towards *C. rectus* GroEL, high resonance unit (RU) values were observed in the saliva samples from patients with periodontitis ($P < 0.01$). Furthermore, the number of teeth with deep pocket depth (≥5 mm) showed a high correlation coefficient with the RU value ($r = 0.50$, $P < 0.01$). *C. rectus* GroEL possessed the ability to stimulate the production of interleukin-6 by gingival fibroblasts. Interestingly, salivary IgA antibody directed to *C. rectus* GroEL caused a partial inhibition of interleukin-6 production. This study showed a relationship between high levels of salivary IgA directed to GroEL and periodontal disease severity. Although additional investigations are required, salivary IgA to GroEL may have a protective role by reducing the inflammatory response induced by GroEL derived from periodontopathogenic bacteria.

Key words: GroEL; periodontitis; salivary immunoglobulin A

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Periodontitis is recognized as a mixed bacterial infection. Gram-negative anaerobic bacteria such as *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* are found in periodontal lesions with alveolar bone loss (36). *Campylobacter rectus*, a gram-negative, anaerobic, asaccharolytic and motile rod-shaped bacterium, has also been implicated in the etiology of several forms of periodontal disease, such as rapidly progressive periodontitis (2) and periodontitis associated with diabetes mellitus or acquired immune deficiency syndrome (34, 35), in addition to adult periodontitis (25). Virulence factors produced by this bacterium have been reported and include cytotoxic activity factors (4) and surface layer (S-layer) protein, which affect the resistance of *C. rectus* to phagocytic uptake and to bactericidal activity of serum (23, 32).

The most common form of periodontitis is the chronic form, which shows a slowly progressive infectious process. Periodontopathogens are subjected to a wide range of stresses that may affect their growth and virulence and that may induce a stress response (1, 15, 18, 24). Heat-shock proteins (HSPs) are constitutively expressed in eukaryotic and prokaryotic cells under normal growth conditions, and exhibit a high level of expression when the cells are subjected to stress conditions. HSPs play a role in the immune response to many bacterial and parasitic pathogens (28). Several groups showed that the bacterial GroEL, belonging to the HSP 60 family, was an immunodominant antigen in patients with periodontitis (14, 19,

29). In addition, Tabeta et al. (29) demonstrated a positive relationship between periodontitis and the level of serum antibody directed to GroEL.

Oral microbial HSPs appear to be important biological molecules because they have the ability to contribute to pathogenic processes (5). We previously showed that *C. rectus* GroEL and *A. actinomycetemcomitans* GroEL have the ability to stimulate the production of interleukin-6 (IL-6) or IL-8 by human gingival fibroblasts/human gingival epithelial cells, and that they were cytotoxic for human gingival epithelial cells when used at a high concentration (6, 11, 31). These results clearly indicated that the GroEL from periodontopathogenic bacteria possesses biological activities and might play a role in the initiation and progression of periodontal disease.

It is well known that salivary immunoglobulin A (IgA) can block the adherence of bacteria to epithelial cells, neutralize bacterial toxins and promote the aggregation of bacteria (20). The levels of salivary IgA directed to the cell extracts of both *P. gingivalis* and *A. actinomycetemcomitans* were significantly higher in subjects with deeper periodontal probing depth compared to control subjects (26). It was reported that the level of specific salivary IgA antibodies against mycobacterial HSP 65 was significantly increased in patients with gingivitis compared to clinically healthy subjects and subjects with periodontitis (27). The aim of this study was to analyze the relationship between periodontal conditions and levels of salivary IgA directed to oral microbial GroEL. In addition, we investigated the potential role of anti-GroEL salivary IgA in patients with periodontitis.

Materials and methods

Study population and clinical parameters

The study population consisted of 63 patients referred to the Clinic of the Preventive Dentistry, The University of Tokushima Hospital, Japan. Each participant completed a medical and dental history and signed an informed consent document for collection of saliva. The inclusion criteria were healthy adult under 65 years old with at least 10 teeth present. Subjects who had received antibiotic treatments within the last 3 months or who showed evidence of systemic disease were excluded from this study. Full mouth periodontal probing depth and the deepest site at each tooth were recorded. After the inserted periodontal probe was removed from the periodontal pocket, the prevalence of teeth with positive bleeding on probing at two sites (buccal and palatal/lingual) per tooth was recorded. Subjects with periodontitis showed the following criteria: presence of a minimum of two deep periodontal probing depths (≥5 mm) on separate teeth, positive for bleeding on probing in deep pocket sites and/or radiographic evidence of alveolar bone loss. Patients without periodontitis were categorized as control subjects in this study.

Determination of salivary IgA concentrations and purification of salivary IgA

The saliva samples collected from subjects were unstimulated, whole mixed saliva. The saliva flow rate of each subject was evaluated by measuring the volume of saliva produced during 5 min. The debris in resting saliva was removed by centrifugation, and the protein concentration was determined by the method of Hartree (7) with bovine serum albumin as the standard. Salivary IgA concentration was measured by enzyme-linked immunosorbent assay according to the method of Ishii et al. (13) with a slight modification. Ninety-six-well microtiter plates were precoated with 100 μl of the goat IgG fraction to human secretory IgA (4.0 μg/ml; Cappel Product, Durham, NC) in 50 mM carbonate–bicarbonate buffer (pH 9.6). The data sheet of this reagent states that there was no reactivity with human IgG, IgM or non-immunoglobulin serum proteins. We also confirmed that this antibody was specific to the α-heavy chain of IgA in our preliminary study. After overnight incubation at 4°C, the plates were washed three times with phosphate-buffered saline (pH 7.4) containing 0.05% Tween-20 (PBST), then blocked with 150 μl Block Ace solution (Dainippon Pharmacology, Kyoto, Japan) for 1 h. After washing with PBST, 100 μl saliva (1 : 1000 dilution) or two-fold serial dilutions of human secretory IgA (39.1 ng/ml to 5000 ng/ml; Cappel Product) was added, and the plates were incubated at 37°C for 1 h. Wells without salivary IgA were used as controls. Bound salivary IgA were detected with horseradish peroxidase (HRP)-conjugated goat anti-human secretory IgA (Cappel Product), possessing the same characteristics as the antibody described above, followed by the addition of 100 μl HRP substrate solution (Bio-Rad, Hercules, CA). After 5 min, the reaction was stopped with 1 molar H_2SO_4 (100 μl/well) and the absorbance at 450 nm was measured with a microplate reader (Bio-Rad).

The purification step of human salivary IgA was as follows. Approximately 10 ml of the resting saliva from a volunteer was dialyzed against 0.01 M, pH 8.0, phosphate buffer for 20 h and then applied to a diethylaminoethyl Sephacel (LKB-Pharmacia, Uppsala, Sweden) column equilibrated in the same buffer. The pass through the IgG-rich fractions was discarded and IgA-rich fractions were eluted with 0.05 M NaCl in 0.01 M phosphate buffer, pH 8.0. The salivary IgA-rich fractions were further purified by passing them over an anti-human IgA (α-chain-specific) agarose column (Sigma, St Louis, MO). After washing the column with phosphate buffer, pH 8.0, the salivary IgA was eluted from the column with 3 M citric acid, pH 3.0. The eluted fractions were passed over a disposable PD-10 Sephadex G-25M column equilibrated with phosphate buffer, pH 8.0, to exchange the buffer according to the manufacturer's instruction (LKB-Pharmacia). Purified salivary IgA was investigated by Western immunoblot using HRP-conjugated goat anti-human salivary IgA and HRP-conjugated anti-secretory component (Nordic Immunological Laboratories, Tilburg, the Netherlands).

Preparation of GroEL proteins from periodontopathogens

The GroEL protein was purified from three species of periodontopathogenic bacteria (*C. rectus* ATCC 33238, *P. gingivalis* ATCC 33277, *A. actinomycetemcomitans* ATCC 29522) and from *Escherichia coli* ATCC 11303 according to the method previously described (9, 11). The purification of the native GroEL protein from bacterial cells following heat-shock treatment at 44°C for 1 h was carried out as follows. The fraction containing the GroEL protein was obtained by affinity chromatography on adenosine 5′-triphosphate (ATP)-agarose (Sigma Chemical Co.). Pooled fractions were concentrated, then the sample investigated using high-performance liquid chromatography on a Superose 6 column (Amersham Pharmacia Biotech Ltd, Uppsala, Sweden) that had been equilibrated with PBS. The eluted fractions were analyzed for the GroEL-like protein by Western immunoblotting as described below and the homology was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and silver nitrate staining. Purified samples in the fractions were collected and stored at −20°C until used. The recombin-

ant human HSP 60 was purchased from StressGen (Victoria, BC, Canada). Before use for stimulation of fibroblasts, the GroEL fractions were applied to a Detoxi-Gel Affinity Pack column (Pierce Biotechnology Inc, Rockford, IL) to remove lipopolysaccharide contamination and the absence of endotoxin was confirmed by a *Limulus* amebocyte lysate, Pyrotell® (Associates of Cape Cod Inc., Woods Hole, MA).

SDS–PAGE and Western immunoblotting analysis

For SDS–PAGE analysis, bacterial GroEL, human HSP 60 or washed bacterial cells were treated at 100°C for 5 min in 62.5 mM Tris–HCl buffer (pH 6.8) containing 1% SDS, 10% glycerol and 5% 2-mercaptoethanol. Bacterial cells were sonicated for 5 min before treatment. SDS–PAGE was performed using 12% polyacrylamide slab gels, and the migrated proteins were transferred (250 mA for 1 h) onto a nitrocellulose membrane. After the unreactive sites were blocked with 3% bovine serum albumin, the membrane strips of bacterial GroEL and human HSP 60 were incubated with saliva (1 : 10 dilution) from each subject. The membrane strips were further incubated with HRP-conjugated goat anti-human secretory IgA (1 : 500 dilution) for 1 h, and then developed with an HRP conjugate substrate kit (Bio-Rad). For the investigation of immunoreactivity against bacterial cell extracts, the selection of samples to be analyzed was based on the reactivity of the saliva with microbial GroEL. Four equivalent volumes of saliva from the four periodontitis patients were mixed, and then used as the first antibody on Western immunoblots according to the procedure described above. The positive immunoreaction with saliva was defined by comparing it with the reactivity of the membrane strip without saliva used as first antibody.

Biospecific interaction analysis by BIACORE 1000

The sensor chip CM5 was activated according to the recommendations of the BIACORE 1000 amine coupling kit, and 20 μg/ml anti-secretory IgA antibody was then covalently coupled to the sensor chip. Saliva samples were adjusted to 20 μg/ml salivary IgA in running buffer (10 mM HEPES, 150 mM NaCl, 0.05% Tween-20 and 3 mM EDTA, pH 7.4). Purified *C. rectus* GroEL (100 μg/ml) and anti-*C. rectus* GroEL polyclonal antibody (pAb-*C. rectus* GroEL, 1 : 500 dilution) (12) were also prepared in the above running buffer. The analyses were performed by injection of the saliva sample, *C. rectus* GroEL then pAb-*C. rectus* GroEL according to the manufacturer's instructions. The resonance signal was recorded continuously during passage of the sample. The difference between the resonance signals measured before saliva sample injection and after pAb-*C. rectus* GroEL injection was related to the amount of bound salivary IgA directed to *C. rectus* GroEL and was expressed as resonance units (RU).

Determination of IL-6 production by Gin-1 cells stimulated with *C. rectus* GroEL and an inhibitory assay of salivary IgA

The human gingival fibroblast cell line Gin-1 was obtained from the American Type Culture Collection (Manassas, VA). Gin-1 cells were seeded into a 24-well plate in supplemented Dulbecco's Modification of Eagle's Medium and cultivated until a confluent monolayer was constituted. One milliliter of the sample containing 2.2 μg/ml of *C. rectus* GroEL in Dulbecco's Modification of Eagle's Medium was added to the confluent monolayer. Gin-1 cells were further incubated at 37°C in a CO_2 incubator (5% CO_2 and 95% air) for 24 h. The IL-6 contents in the culture supernatants derived from stimulated Gin-1 cells were measured using a commercial assay kit, Human IL-6 Cyto-Sets™ (BioSource International Inc., Camarillo, CA) according to the manufacturer's protocol. For inhibitory experiments, *C. rectus* GroEL was preincubated with the prepared salivary IgA solution (3.5 μg/ml) or PBS as control at 37°C for 30 min, and then Gin-1 cell cultures were stimulated. All experiments were performed in triplicate.

Statistical analysis

The data were expressed as mean ± standard deviation for all the parameters measured. The statistical analysis was performed with commercially available statistical package, SPSS Ver.11.0J (SPSS Inc., Chicago, IL). The characteristics of subjects with periodontitis and control subjects were compared using Mann–Whitney *U*-test. The positive immunoreactivity of salivary IgA against microbial GroEL/human HSP 60 between two groups was analyzed using a chi-squared test or Fisher's exact test. Spearman's correlation coefficients (two-tailed) were calculated to determine the association of the RU value of salivary IgA and clinical parameters related to periodontal conditions. Mann–Whitney *U*-test was also used to compare IL-6 production and the inhibitory assay of salivary IgA.

Results

The clinical characteristics of the selected subjects are presented in Table 1. The subjects had an average age of 44.5 years and a mean of 25.4 teeth; there was no difference between the periodontitis and control groups. The means ± standard deviation of the number of teeth with a periodontal probing depth ≥ 4 mm in subjects with periodontitis and control subjects were 15.1 ± 6.5 and 1.4 ± 1.5, respectively. There was no significant difference in the parameters related to saliva characteristics between the two groups.

The summary of salivary IgA immunoreactivity against bacterial GroEL and human HSP 60 is presented in Table 2 and the typical immunoreaction profile of salivary IgA from periodontitis subjects is shown in Fig. 1. Among 35 subjects with periodontitis, salivary IgA antibodies from 22 subjects reacted positively with at least

Table 1. Characteristics of subjects with periodontitis and control subjects

Characteristics	Subjects with periodontitis	Control subjects	
Number of subjects (male/female)	35 (14/21)	28 (7/21)	
Age (years)	46.5 ± 10.3	42.1 ± 10.7	NS
Number of present teeth	25.3 ± 3.7	25.6 ± 2.3	NS
Number of teeth with PPD (≥4 mm)	15.1 ± 6.5	1.4 ± 1.5	**
Number of teeth with PPD (≥5 mm)	5.7 ± 6.0	0.1 ± 0.3	**
Percent teeth with BOP	79.0 ± 21.7	36.9 ± 23.5	**
Saliva flow rate (ml/5 min)	1.58 ± 1.12	1.41 ± 0.87	NS
Protein (mg/ml)	3.20 ± 2.07	2.55 ± 1.88	NS
Salivary IgA (mg/ml)	0.26 ± 0.19	0.25 ± 0.16	NS

BOP, bleeding on probing; PPD, periodontal probing depth.
Each value represented the mean ± SD.
NS, not significant; *$P < 0.05$; **$P < 0.01$, by Mann–Whitney *U*-test.

Table 2. Immunoblot profiles of salivary IgA in saliva from subjects with periodontitis

Sex	F	F	F	F	M	F	F	M	M	M	M	M	M	M	F	F	F	F	F	M	F	F	F	M	F	M	F	F	F	M	M	F	F	F	No. of positive
Age	23	28	29	29	30	33	36	40	41	43	44	44	44	44	45	45	49	49	50	50	50	51	51	51	53	53	54	55	55	57	58	59	60	62	62	reactions
Subjects with periodontitis ($n = 35$)																																				
*A.a.*GroEL	+	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	6
*C.r.*GroEL	−	+	−	−	−	−	+	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	+	−	−	+	+	+	−	−	+	−	+	+	11
*E.c.*GroEL	−	−	−	+	−	−	−	−	−	−	−	−	+	−	−	+	−	−	+	−	−	+	−	+	−	−	+	+	−	−	−	−	−	+	+	10
*P.g.*GroEL	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	+	6
HuHSP60	−	−	+	−	−	−	−	−	+	−	−	−	+	+	+	−	−	+	+	−	−	+	−	−	−	+	−	+	−	−	+	−	−	−	+	12
WIB (Fig. 1)							†						†	†										†												
Biacore exp.		*			*		*	*			*	*	*	*		*	*		*	*	*	*		*	*	*	*								*	

Sex	M	F	F	M	F	F	F	F	F	F	F	M	F	F	F	F	F	F	F	M	F	F	F	F	M	F	M	M	F	No. of positive
Age	26	28	29	29	30	30	31	32	33	34	35	37	43	44	45	46	46	47	48	49	50	51	51	53	54	57	58	58	63	reactions
Control subjects ($n = 28$)																														
*A.a.*GroEL	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+	−	+		4
*C.r.*GroEL	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−		2
*E.c.*GroEL	+	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	+	−	−	−	+		7
*P.g.*GroEL	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−		0
HuHSP60	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	+	+	−	−	−	−		4
Biacore exp.	*	*	*				*			*	*		*	*	*		*	*		*	*	*	*	*			*			

Results of the immunoblotting analysis were expressed as follows: +, positive reaction; −, negative reaction.
A.a., *Actinobacillus actinomycetemcomitans*; *C.r.*, *Campylobacter rectus*; *E.c.*, *Escherichia coli*; *P.g.*, *Porphyromonas gingivalis*; Hu, human; WIB, Western immunoblotting.

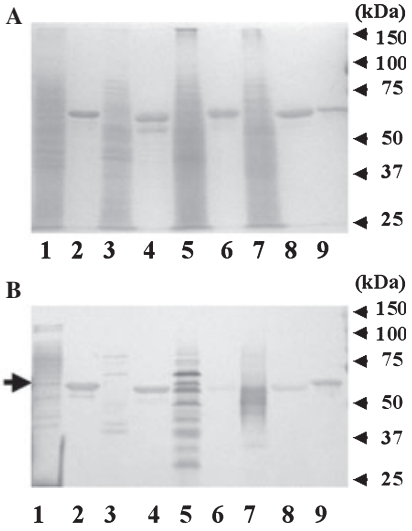

Fig. 1. Typical immunoreactivity of microbial GroEL/human HSP 60 and bacterial cell extracts with salivary IgA from subjects with periodontitis. Saliva samples used for this experiment are presented in Table 2 as daggers. Five micrograms of cell proteins and 0.1 μg of GroEL were separated by SDS–PAGE, then transferred to nitrocellulose membrane. (A) SDS–PAGE stained with Coomassie brilliant blue, (B) Western immunoblots revealed by salivary IgA. Lanes 1 and 2, *C. rectus* cell extracts and GroEL; lanes 3 and 4, *A. actinomycetemcomitans* cell extracts and GroEL; lanes 5 and 6, *P. gingivalis* cell extracts and GroEL; lanes 7 and 8, *E. coli* cell extracts and GroEL; lane 9, human HSP 60.

one HSP antigen. The positive reactions were observed in both men and women, and also in a variety of ages. The relationship between a positive reaction and salivary flow rate or salivary concentration was analyzed, and no significant difference was observed (data not shown). Positive reactions with three or more antigens were found in seven subjects. Significant differences were observed in the positive reactions of salivary IgA directed to *C. rectus* GroEL or *P. gingivalis* GroEL between subjects with periodontitis and control subjects ($P < 0.05$). Reactions directed to human HSP 60 were observed in 34% of subjects with periodontitis although this was not significant. Mixed saliva samples used as the first antibody on Western immunoblots were obtained from four periodontitis patients and are indicated in Table 2 by daggers. Immunoreactive bands were detected in all bacterial cell extracts used. The *C. rectus* cell extract (lane 1 in Fig. 1) and *P. gingivalis* cell extract (lane 5) reacted relatively strongly with salivary IgA when compared with the cell extracts of *A. actinomycetemcomitans* and *E. coli*. Among the immunoreactive bands present in the *C. rectus* cell extract, the 64-kDa band shown by an arrow in lane 1 also reacted with pAb-*C. rectus* GroEL (data not shown). The low reactivity seen with *P. gingivalis* GroEL (lane 6) is not surprising considering that four other positive reactions in subjects were also weak compared to the other GroEL/HSP 60.

By using the BIACORE system, the antigen (*C. rectus* GroEL)–antibody (salivary IgA) interaction and the parameters related to periodontal disease were investigated. Resting saliva samples of sufficient volume to be used in this experiment are indicated in Table 2 by asterisks. The numbers of subjects belonging to the periodontitis group and control group were 19 and 17, respectively, and no difference was observed between the two groups except for the parameters related to periodontal conditions (data not shown). The RU value obtained by BIACORE assay correlated significantly with the positive immunoreaction of IgA antibodies to GroEL assayed by Western immunoblot ($P < 0.01$, Spearman's rank test). The mean RU values in the periodontitis group (47.5 ± 6.2 RU) were significantly higher than those in the control group (37.1 ± 10.9 RU). There was a significant positive relationship between the RU value and the number of teeth with deep periodontal probing depth in the 36 subjects measured (Fig. 2). The correlation coefficients of the number of teeth with periodontal probing depth ≥ 4 mm (Fig. 2A) and with periodontal probing depth ≥ 5 mm (Fig. 2B) were 0.477 and 0.502, respectively, and both values were significant ($P < 0.01$). The percentage of teeth positive for bleeding on probing (% bleeding on probing) was also significantly correlated with the RU value ($r = 0.358$, $P < 0.05$) (data not shown).

The production of IL-6 by Gin-1 cells stimulated with the *C. rectus* GroEL and the inhibitory effects of salivary IgA were investigated. When the Gin-1 cells were incubated with *C. rectus* GroEL at a concentration of 2.2 μg/ml, no differences in cell viability were found between the treated cells and the control cells (data not shown). The saliva used for the inhibitory

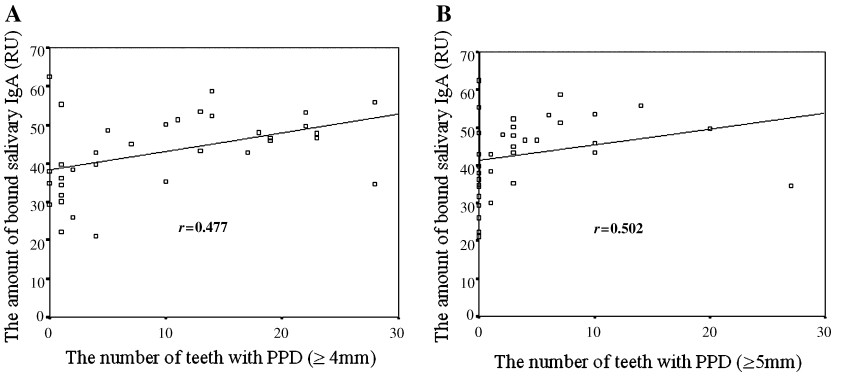

Fig. 2. Relationship between the titer of salivary IgA directed to *C. rectus* GroEL and clinical parameters related to periodontitis. Saliva samples used for this experiment are presented in Table 2 as asterisks. (A, B) show the RU value vs. the number of teeth with periodontal probing depth (≥4 mm) and the number of teeth with periodontal probing depth (≥5 mm), respectively. Each solid line and *r* were the regression line and correlation coefficient, respectively.

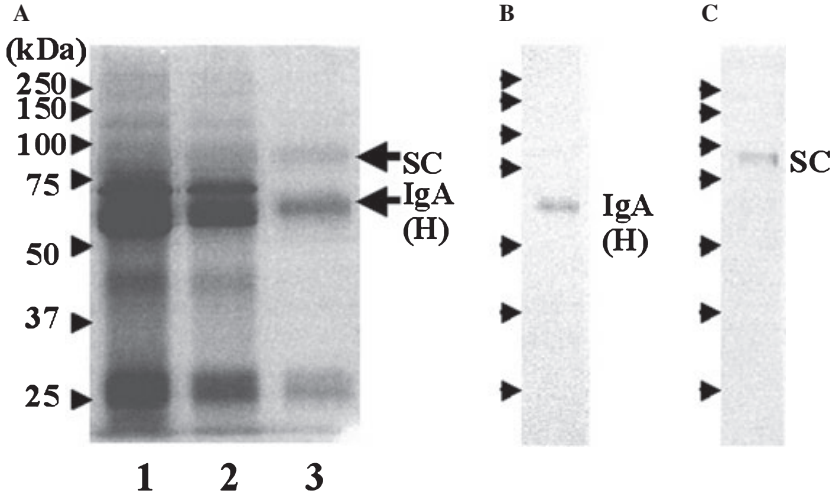

Fig. 3. SDS–PAGE and Western immunoblot of purified salivary IgA used in the inhibition assay. (A) SDS–PAGE stained with silver nitrate staining, (B) Western immunoblots of purified salivary IgA (0.2 μg) revealed by anti-human secretory IgA (α-heavy chain specific), (C) Western immunoblots of purified salivary IgA (0.2 μg) revealed by anti-secretory component. Lanes 1, 2 and 3 in (A) were original saliva (3.0 μg), sample after ion-exchange chromatography on diethylaminoethyl Sephacel (1.5 μg) and purified salivary IgA (0.3 μg). The values on the left in (A) are molecular size and the arrow in (B, C) show the same molecular size in (A).

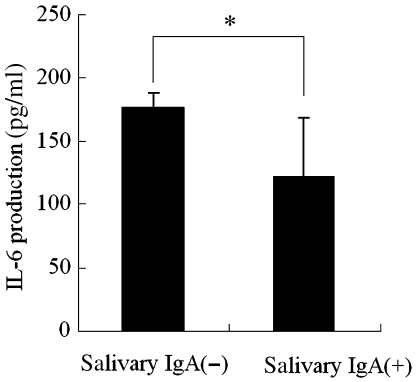

Fig. 4. Production of IL-6 by Gin-1 cells stimulated with *C. rectus* GroEL in the absence of salivary IgA [salivary IgA (−)] and in the presence of a high titer of salivary IgA [salivary IgA (+)]. Each column represents the mean ± standard deviation of three experiments. Asterisks indicate statistically significant differences (*$P < 0.05$).

assay was immunoreactive with *C. rectus* GroEL by Western immunoblot and possessed a 45.5 RU value determined with the BIACORE assay. The fractions obtained from the purification procedure were analyzed by SDS–PAGE and silver nitrate staining (Fig. 3A). The purification procedure yielded 36 μg salivary IgA from 10 ml saliva. Using Western immunoblot analysis, the purified sample was shown to comprise an α-heavy chain of IgA (Fig. 3B) and a secretory component (Fig. 3C). The difference in the amount of IL-6 produced by cells treated with GroEL and control cells was found to be 176.5 g/ml (Fig. 4). When *C. rectus* GroEL was pretreated with purified salivary IgA, the difference between IL-6 production by stimulated Gin-1 cells and control cells was only 121.5 pg/ml. By comparing these data, the release of IL-6 by Gin-1 cells was found to be inhibited (Mann–Whitney *U*-test, $P < 0.05$) by approximately 31%.

Discussion

Associations between the host response and severity of disease have been investigated for a variety of infectious diseases. The immunological diagnosis of disease requires antigens with high specificity and immunogenicity. Several investigators demonstrated that HSPs are immunodominant antigens and can be detected in diseased periodontal sites. Lopatin et al. studied a HSP 90 protein in *P. gingivalis*, which was detected in extracellular vesicles, and showed the presence of antibodies in the sera from periodontitis patients that cross-react with this protein (16, 17). These authors suggested that tests could be developed to evaluate the risk for periodontitis (16, 17). In this study, we investigated whether the measurement of the salivary IgA response directed to GroEL from periodontopathogenic bacteria might be a method of diagnosing periodontitis.

It is well known that whole saliva within the oral cavity receives antibodies from three sources: salivary glands, the gingival crevice, and antibody-forming plasma cells in the oral mucosa. Furthermore, IgA in saliva is predominantly secretory IgA from the salivary glands whereas the main source of IgG and IgM in whole saliva is from gingival fluid (33). Minor contamination of IgA with periodontal pocket fluid may exist in the saliva samples used in this study. GroEL proteins belonging to the HSP 60 family have been considered as key elements in the pathogenesis of several bacterial infections (28). Since GroEL proteins from periodontopathogenic bacteria are well conserved and their immunoreactivities resemble each other more closely than those of other HSPs (10, 11), we chose to study this protein. We found that there were differences in the immunoreactive patterns of salivary IgA against five types of antigen. Among the bacterial GroEL used, *C. rectus* GroEL from periodontitis subjects showed significant differences for positive reactions with salivary IgA from that of control subjects

(corresponding to the group of healthy/slight gingivitis subjects). These results differed from those reported by Schett et al. (27). They examined anti-mycobacterial HSP 65, *E. coli* GroEL and human HSP 60 in saliva (salivary IgA) and found that the antibody concentrations in subjects with gingivitis were higher than those found in healthy subjects and patients with periodontitis. Discrepancies in our observations showing that the ratio of positive immunoreaction and the RU value in periodontitis patients were higher than those in control subjects may be explained by the fact that we used GroEL from periodontopathogenic bacteria, which may differ from other bacterial GroEL. In addition, we have demonstrated previously that GroEL protein and S-layer protein of *C. rectus* may share the same epitope as immunodominant antigen (12). Doublet immunoreactive bands with approximately 120–150 kDa, which may correspond to the S-layer protein, were detected in *C. rectus* cell extracts (lane 1 in Fig. 2). Therefore, S-layer protein might affect the immunological reaction of salivary IgA directed to *C. rectus* GroEL.

The *P. gingivalis* GroEL also showed significant differences in its reactivity, 17% (6/35) of the saliva samples were positive in the periodontitis group. Tabeta et al. (29) demonstrated that the number of periodontitis patients showing a positive response to *P. gingivalis* GroEL was higher than that of healthy subjects by Western immunoblot analysis using IgG from the sera. These results support the tendency for the specificity of anti-*P. gingivalis* GroEL in patients with periodontitis even if the characteristics of the antibody are different between serum IgG and salivary IgA. On the other hand, *A. actinomycetemcomitans* GroEL was reported to be surface associated (21), and the antibodies reactive to *A. actinomycetemcomitans* GroEL in periodontitis patients might be induced by the cross-reactivity with the HSP 60 proteins of other bacteria (22). Tabeta et al. (30) reported that the serum antibody level directed to *A. actinomycetemcomitans* GroEL tended to be higher in periodontitis patients than in healthy subjects although no significant difference was observed. In our study, salivary IgA directed to *A. actinomycetemcomitans* GroEL from six of 35 periodontitis patients showed positive reactions, but no significant difference was observed between the two groups.

The *C. rectus* GroEL was selected for a detailed analysis on the basis of its reactivity in the BIACORE system because the positive reactions against *C. rectus* GroEL on Western immunoblot were the most prevalent and often gave the strongest reactivity (data not shown). A significant positive relationship between the RU values and the number of teeth with deep periodontal probing depth was observed. These results indicate that the measurement of salivary IgA titers directed to HSPs of periodontopathogenic bacteria should be considered to be a valid assay for the prediction of periodontal condition. Mycobacterial GroEL has a high specificity as a marker for Behçet's disease and may be of interest in the development of a diagnostic test using the blood from a patient (8). Saliva samples are advantageous for the screening of periodontal condition because, compared to blood samples, they are easy to obtain from patients.

The mechanisms of tissue destruction in chronic periodontal disease have not been defined clearly, though several inflammatory cytokines such as IL-6 and IL-8 are considered to be involved. Bacterial HSPs may have deleterious effects on host cells and deserve to be considered as a virulence factor. We demonstrated that low concentrations of native *C. rectus* GroEL enhance IL-6 secretion by human epithelial cells, while high concentrations produce toxic effects (31). Galdiero et al. (3) showed that *E. coli* GroEL possesses the capacity to induce the production of IL-6 by human monocytes and secretion was inhibited by a specific monoclonal antibody against GroEL. Although minor salivary components may be involved, the 31% decrease in IL-6 secretion by Gin-1 cells stimulated with *C. rectus* GroEL obtained in the presence of salivary IgA seemed to be related to modulation of the cell interactions with *C. rectus* GroEL. This result indicated that salivary IgA might be protective with regard to the inflammatory response induced by *C. rectus* GroEL. Further research is needed to clarify the definitive molecular mechanisms leading to the effects observed in this study.

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