Humoral and cellular immune responses to the fimbriae of *Porphyromonas gingivalis* and their synthetic peptides

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Summary. Subcutaneous injection of fimbriae from *Porphyromonas gingivalis* strain 381 in Freund's incomplete adjuvant (FIA) resulted in an excellent serum anti-fimbrial immunoglobulin G (IgG) response in guinea-pigs and BALB/c mice. Administration of *P. gingivalis* fimbriae also elicited distinct cellular immune responses to the fimbriae in terms of ear lobe reaction in BALB/c but not in BALB/c *nu/nu* mice, and of skin reaction in guinea-pigs. When the guinea-pigs were given a semi-synthetic adjuvant GM-53—sodium β -N-acetylglycosaminyl-(1 $\rightarrow$ 4)-N-acetylmuramyl-L-alanyl-D-isoglutaminyl-(L)-stearyl-(D)-meso-2, 6-diaminopimelic acid-(D)-amide-D-alanine—and fimbriae in FIA by subcutaneous injection, more enhanced production of serum anti-fimbrial IgG and stronger cellular immune responses were induced in the guinea-pigs than in those given fimbriae alone. Synthetic peptide FP381(202–221), which corresponds to the amino-acid residue numbers 202–221 based on the amino-acid sequence of fimbrilin from *P. gingivalis* strain 381, elicited humoral and cellular immune responses in guinea-pigs immunised with the fimbriae or FP381(202–221). Furthermore, subcutaneous administration of synthetic peptide FP381(61–80) with GM-53 induced lesser degrees of humoral and cellular immune responses in guinea-pigs than did FP381(202–221). However, when the fimbriae or FP381(61–80) were administered with bovine serum albumin (BSA), markedly elevated levels of specific anti-BSA antibody were seen in the serum of BALB/c mice. These results clearly indicated that fimbriae from *P. gingivalis* 381 and their oligopeptide segments induced humoral and cellular immune responses and exhibited immuno-adjuvant activities in guinea-pigs and BALB/c mice.

Introduction

Porphyromonas gingivalis is a gram-negative, black-pigmented anaerobic bacterium that has been associated with the development of chronic periodontal diseases in man.¹ Antigen-specific antibodies to the surface components of *P. gingivalis* have been detected in the serum and crevicular fluid of patients with periodontal diseases.^{2–6} Among these components, the fimbriae of *P. gingivalis* have been presumed to be important for the adherence of the organisms to host cells.^{7–8} We have shown previously that *P. gingivalis* fimbria-specific antibody-secreting cells are increased in the inflamed gingival tissues of patients with periodontal diseases, and antigen-specific antibodies are present in their serum.⁹ It has been shown also that subcutaneous or oral immunisation with *P. gingivalis* fimbriae in BALB/c mice induces markedly increased levels of fimbria-specific IgA or IgG antibodies, or both, in saliva and serum, and fimbria-specific antibody-producing cells have been detected in various lymphoid tissues.^{10, 11}

Fimbriae from *P. gingivalis* strain 381 and their synthetic peptide segments are known to have multi-

functional properties such as antigenicity, haemagglutinating activity, mitogenic and polyclonal B-cell activation (PBA) activity for mouse splenocytes and induction of production of cytokines such as tumour necrosis factor- α (TNF- α) and interleukin-6 (IL-6) in human monocyte/macrophage cultures.¹² The present study was designed to determine if *P. gingivalis* fimbriae and their synthetic peptide segments known to exert various immunobiological effects in a host would induce humoral and cellular immune responses and exhibit immuno-adjuvant activity in guinea-pigs and mice.

Materials and methods

Animals

Male BALB/c and BALB/c *nu/nu* mice (8 weeks old) were obtained from Japan SLC, Inc., Hamamatsu, Japan. Closed-colony male albino guinea-pigs (weight 300–350 g) were purchased from Nihon Rabbit Inc., Osaka, Japan.

Preparation of fimbriae from *P. gingivalis* 381

P. gingivalis strain 381 was grown anaerobically in GAM broth (Nissui, Tokyo, Japan) supplemented

with haemin and menadione for 26 h at 37°C, and its fimbriae were isolated as described previously.¹⁰ No contaminating proteins were detected by SDS-PAGE, and the subunit structure of fimbriae, fimbrilin, was identified as a single band of the 41-kDa subunit protein.¹⁰

Synthesis of peptide

Peptides FP381(61–80), whose amino-acid sequence is GKT LAEVKALTTELTAENQE, and FP381(202–221), which corresponds to VAPAADAPQGFTVL-ENDYSA, based on the amino-acid sequence of the native fimbrilin of *P. gingivalis* 381 derived from the nucleotide sequence,¹³ were synthesised with a model 9050 peptide synthesiser (Japan Millipore Ltd, Tokyo) by the solid-phase methods.¹⁴ After cleavage of the protecting groups with trifluoroacetic acid:*m*-cresol:ethanedithiol:methanesulphonic acid (90:2.5:5:2.5), the peptide was purified by high performance liquid chromatography on a reversed phase column (Capcell Pak C18 SG120, 1.5 × 15 cm; Shiseido, Tokyo) with a linear gradient (6–60%) of acetonitrile in acetic acid 0.1% at a flow rate of 23 ml/min. The major peak fraction of the peptide was collected by monitoring the absorbance at 220 nm. The purified peptide synthesised was analysed for composition by quantitative amino-acid analysis and sequenced by automated Edman degradation with a model 6400/6600 protein sequencer (Japan Millipore Ltd).

Adjuvant

Muramyldipeptide (MDP) and sodium β -*N*-acetylglucosaminyl-(1 → 4)-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-(L)-stearoyl-(D)-*meso*-2, 6-diaminopimelic acid-(D)-amide-D-alanine (GM-53)¹⁵ were generously supplied by Daiichi Seiyaku Co., Tokyo and Dainippon Pharmaceutical Co., Osaka, respectively. Freund's incomplete adjuvant (FIA) was purchased from Difco.

Assay for humoral and cellular immune responses

Groups of six guinea-pigs were given various doses of fimbriae on their synthetic peptides by injection into the left hind footpad with or without a dose of adjuvant GM-53 in FIA on day 0. Skin tests were performed to evaluate the induction of a cellular immune response to fimbrial antigen or the synthetic peptide segments of the fimbriae.¹⁶ Briefly, the immunised guinea-pigs were given either an intracutaneous injection of 100 µg of fimbriae or of their synthetic peptide segments 35 days after immunisation. The skin test was examined for the extent and degree of redness, swelling and necrosis 48 h after the intradermal provocative injection. Serum specimens were taken 1 day after the skin test and stored at –80°C until assayed. The immunoglobulin G (IgG) levels of antigen-specific antibodies in the serum of the guinea-pigs were determined by an ELISA technique.¹⁰ The individual

values are expressed as points and the bars indicate the median values.

In some experiments, groups of 10 BALB/c and BALB/c *nu/nu* mice were given 100 µg of the fimbriae in FIA by subcutaneous injection on days 0 and 28. On day 33 these mice were given 10 µg of the fimbriae by intracutaneous injection into the ear lobe, whose thickness at the injection site was measured daily for 3 days with a dial gauge (Model G; Ozaki Corp., Tokyo). Serum specimens were taken 1 day after the ear lobe test. Antigen-specific IgG levels were determined by ELISA.¹⁰

Immuno-adjuvant activity in mice

BALB/c mice (10/group) were immunised by subcutaneous injection of 100 µg of bovine serum albumin (BSA; Sigma) with or without 100 µg of test specimen in PBS or FIA, or were given FIA alone, on days 0 and 28. Serum anti-BSA IgG antibody levels were determined by ELISA 5 days after the secondary immunisation.

Statistical analysis

The significance of differences between means of groups was determined by Student's *t* test.

Results

*Induction of humoral and cellular immune responses in guinea-pigs and BALB/c mice after immunisation with *P. gingivalis* fimbriae*

Guinea-pigs immunised with *P. gingivalis* 381 fimbriae in FIA were found to have raised anti-fimbrial IgG antibody levels in their serum (fig. 1). Subcutaneous injection of *P. gingivalis* fimbriae in FIA resulted in a dose-dependent induction of serum antibody responses over a concentration range of 50–500 µg of fimbriae/animal. The production of fimbria-specific IgG reached its maximum at 500 µg and was reduced at 1000 µg/animal. Furthermore, the addition of a semi-synthetic adjuvant, GM-53, a stearoyl derivative of the disaccharide tetrapeptide isolated from *Lactobacillus plantarum* cell walls, resulted in much higher levels of fimbria-specific IgG responses in serum (fig. 1). The greatest adjuvant effect was observed when GM-53 was given subcutaneously to guinea-pigs at the maximum dosage of 500 µg/animal with an equal dose of fimbriae. On the other hand, whereas *P. gingivalis* fimbriae also induced anti-fimbrial IgG in serum after immunisation in BALB/c mice, they did not do so in BALB/c *nu/nu* mice (data not shown).

P. gingivalis fimbriae could induce a cellular immune response in guinea-pigs when administered subcutaneously with or without adjuvant GM-53 in FIA. Fig. 2 illustrates the skin reaction after intradermal

Fig. 1. Dose-response relationship between *P. gingivalis* fimbriae and serum IgG titres in guinea-pigs. Seven groups of six guinea-pigs were immunised subcutaneously with various doses of fimbriae with or without 500 µg of GM-53 in FIA, with GM-53 in FIA or with FIA alone on day 0. Serum anti-fimbrial IgG levels were determined by ELISA 1 day after skin test (on day 38). The individual values are expressed as points and the bar indicates the median value.

Fig. 2. Cellular immune responses induced by *P. gingivalis* fimbriae in seven groups of guinea-pigs (see fig. 1). Skin reaction was determined 35 days after immunisation. The individual values are expressed as points and the bar indicates the median value.

challenge of guinea-pigs immunised with fimbriae with or without GM-53 in FIA or FIA alone. A clear difference in the redness and swelling of the skin was noted between the immunised groups and the control group. *P. gingivalis* fimbriae were clearly found to induce a skin reaction in guinea-pigs and a linear dose-reaction relationship was seen between the dose of fimbriae and the degree of this skin reaction and presence of necrosis (fig. 2). In animals given fimbriae together with adjuvant GM-53 (500 µg) the swelling at the challenge site was more severe than in animals given an equal dose of fimbriae alone (fig. 2). On the

other hand, whereas *P. gingivalis* fimbriae induced marked ear lobe reactions in BALB/c mice on intra-dermal challenge, they did not do so in BALB/c nu/nu mice (fig. 3).

Induction of humoral and cellular immune responses in guinea-pigs after immunisation with synthetic peptide of fimbriin

Synthetic peptide segments FP381(61–80) and FP381(202–221) were investigated for induction of humoral and cellular immune responses in guinea-

Fig. 3. The time course of the ear lobe reaction to fimbrial provocative injection in (a) BALB/c and (b) BALB/c nu/nu mice given *P. gingivalis* fimbriae in FIA (●) or FIA alone (○). Results indicate ear lobe reaction after intracutaneous injection of 10 μ l of antigen solution (1 mg/ml). *Statistical difference from the value for the control (FIA alone) group, $p \leq 0.01$.

		Serum IgG concentration (μ g/ml) specific for					
Immunogen	Dose (μ g/animal)	Fimbriae		FP381 (61-80)		FP381 (202-221)	
		0	20	0	20	0	20
FP381(61-80) with GM-53	500	•+		•			
FP381(61-80)	500	•		•			
FP381(202-221) with GM-53	500		•+			•+	
FP381(202-221)	500	•+				•+	
Fimbriae	500		•*			•+	
GM-53	500	•		•		•	
FIA alone		•		•		•	

Fig. 4. Serum immune responses in guinea-pigs immunised with *P. gingivalis* fimbriae and their synthetic peptide segments (see fig. 1). The individual values are expressed as points and the bar indicates the median value. *Anti-fimbrial IgG concentration was 18.6 SE 2.0 mg/ml.

pigs. Subcutaneous injection of FP381(61-80) or FP381(202-221) induced the production of anti-FP381(61-80) or anti-FP381(202-221) IgG antibodies in the serum of guinea-pigs immunised with the synthetic peptide antigen with or without GM-53 in FIA (fig. 4). However, FP381(61-80) was far less effective than FP381(202-221) in inducing the production of serum antigen-specific IgG in guinea-pigs. Furthermore, FP381(202-221) but not FP381(61-80) reacted with the serum of the guinea-pigs immunised with *P. gingivalis* fimbriae in FIA, and similarly the fimbrial antigen reacted with serum IgG specific for FP381(202-221) (fig. 4).

FP381(61-80) did not elicit a significant skin reaction in guinea-pigs immunised with *P. gingivalis* fimbriae or FP381(61-80) in FIA, but did evoke a skin reaction when the peptide immunogen in FIA was combined with GM-53 (fig. 5).

Adjuvant effect of *P. gingivalis* fimbriae and their synthetic peptide

The table shows that *P. gingivalis* fimbriae and FP381(61-80) increased the production of anti-BSA IgG when BALB/c mice were inoculated with BSA and with fimbriae or with FP381(61-80) in PBS or

Fig. 5. Cellular immune responses induced by *P. gingivalis* fimbriae and their synthetic peptide segments in guinea-pigs. Five groups of six guinea-pigs were immunised subcutaneously with the indicated dose of *P. gingivalis* fimbriae or their synthetic peptide segments with or without 500 µg of GM-53 in FIA, with GM-53 in FIA, or with FIA alone on day 0. Skin reaction was determined 35 days after immunisation. The individual values are expressed as points and the bar indicates the median value.

Table. Adjuvant activities of *P. gingivalis* fimbriae and their synthetic peptide

Adjuvant	Immunogen	Mean (SE) serum anti-BSA IgG concentration (mg/ml) after injection of BSA with	
		PBS	FIA
Fimbriae	BSA	0.14 (0.01)†	15.2 (1.1)†
FP381(61-80)	BSA	0.10 (0.02)†	12.2 (1.0)†
MDP	BSA	1.73 (0.28)†	10.8 (0.8)*
None	BSA	0.01 (0.003)	6.1 (0.4)
None	None	0 (0)	0 (0)

MDP, muramyl dipeptide. Statistical difference from the values of the group given BSA alone at * $p \leq 0.05$ and † $p \leq 0.01$, respectively.

FIA. The adjuvant activity of MDP in PBS was significantly greater than that of the fimbriae ($p < 0.0001$) but fimbriae in FIA showed more adjuvancy than MDP in FIA ($p < 0.005$).

Discussion

P. gingivalis cell surface antigens have been found to exhibit strong immune responses in man and animals. Enhanced levels of antibodies specific for these anti-

gens have been detected in the serum and crevicular fluid of patients with chronic periodontal diseases.²⁻⁶ Recently, we have found that the number of *P. gingivalis* fimbriae-specific antibody producing cells were increased markedly in the gingival tissues of patients with advanced adult periodontitis.⁹ We have also reported that raised levels of anti-fimbrial antibodies in serum and saliva and increased numbers of antigen-specific antibody-producing cells in various tissues were observed in BALB/c mice immunised subcutaneously or orally with *P. gingivalis* fimbriae in FIA or liposomes.^{10,11,17} In this study, a cellular as well as a humoral immune response to *P. gingivalis* fimbriae occurred in guinea-pigs and BALB/c mice given the fimbriae with or without a semi-synthetic adjuvant GM-53 in FIA by subcutaneous injection.

Subcutaneous injection of *P. gingivalis* fimbriae together with FIA significantly raised the levels of anti-fimbrial IgG in serum and induced a marked ear lobe reaction to a provocative challenge in BALB/c but not in BALB/c nu/nu mice. However, administration of a protein antigen, BSA or ovalbumin (OA), did not result in a significant ear lobe reaction in BALB/c mice immunised with BSA or OA in FIA (data not shown). These results revealed that *P. gingivalis* fimbriae induce cellular as well as humoral

immune responses, and we suggest that this antigenicity depends on the T cells of BALB/c mice.

Our previous studies indicated that chemically synthesised oligopeptide segments of the fimbrial subunit protein (fimbriin) of *P. gingivalis* strain 381 exhibited a variety of immunobiological activities such as agglutination of rabbit erythrocytes, mitogenicity and PBA activities and induction of cytokines in human monocyte-macrophage cultures.¹² From amongst these synthetic peptides we selected two peptide segments: FP381(202–221), which was one of the immunodominant epitopes of the fimbrial protein as shown by its inhibition of the ELISA reaction for rabbit IgG specific for *P. gingivalis* fimbriae, and FP381(61–80) that agglutinated rabbit erythrocytes, was mitogenic and caused PBA of BALB/c splenocytes. These peptides were found to induce cytokines such as TNF- α and IL-6 production in human monocyte-macrophage cultures.¹² ELISA reaction revealed that levels of FP381(202–221)- but not FP381(61–80)-specific IgG were elevated in serum from guinea-pigs which had been immunised with *P. gingivalis* fimbriae (fig. 4). Fimbriae also reacted with serum from guinea-pigs immunised with FP381(202–221). Furthermore, FP381(61–80) and FP381(202–221) were themselves antigenic for guinea-pigs. How-

ever, these peptides did not elicit a positive skin reaction in guinea-pigs immunised with the fimbrial antigen, whereas immunisation with FP381(61–80) only led to a skin reaction when given together with GM-53 (fig. 5). FP381(202–221) induced a positive skin reaction when injected with or without GM-53. These results suggest that FP381(202–221) is the segment that contains an important antigenic epitope and is capable of inducing humoral immune response to *P. gingivalis* without showing other activities.

FP381(61–80) was shown to exhibit enough adjuvanticity to stimulate a humoral immune response to BSA in BALB/c mice when injected with BSA and FP381(61–80) in PBS or FIA (table). The ability of FP381(61–80) to exert this adjuvant effect was comparable to that of a synthetic adjuvant, MDP. The powerful antigenicity of *P. gingivalis* fimbriae may be attributed to adjuvant-active region(s) within the fimbrial molecules. Finally, a semi-synthetic adjuvant GM-53 was shown to stimulate potently both humoral and cellular immune responses to *P. gingivalis* fimbriae and their synthetic peptide antigens in this study, indicating that synthetic peptides such as FP381(202–221) conjugated with GM-53 or FP381(61–80) as an adjuvant may be useful as suitable vaccine candidates against *P. gingivalis* infection.

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