HOST RESPONSE TO INFECTION

The potential protective immune responses to synthetic peptides containing conserved epitopes of *Porphyromonas gingivalis* fimbrial protein

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Summary. The immunodominant and T-cell epitopes within the fimbrial subunit protein (fimbriin) of *Porphyromonas gingivalis* strain 381 were analysed by multi-pin peptide synthesis technology. Six regions with immunodominant epitopes within a sequence of 337 amino acids that reacted with the serum of patients with adult periodontitis were detected. T cells from mice immunised with *P. gingivalis* fimbriae exhibited proliferative responses to *P. gingivalis* fimbriae or to six 10-mer synthetic peptides from the amino-acid sequence of the fimbriin. Three synthetic peptides that contained the regions responsible for the immunodominant epitopes as well as those which coincided with a T-cell epitope of *P. gingivalis* fimbrial molecules—FP381(142–161), FP381(202–221) and FP381(216–243)—were selected and synthesised. When guinea-pigs were immunised with fimbriae or one of the three synthetic peptide segments and an adjuvant in Freund's incomplete adjuvant, enhanced production of the antigen-specific IgG antibodies was induced in the serum of the animals. Furthermore, of the three synthetic peptides tested, FP381(202–221) produced the greatest protective immune response in guinea-pigs infected with *P. gingivalis* and this was more effective than the native fimbrial protein.

Introduction

The protective immune responses to pathogens in infectious diseases are generally recognised as involving the induction of both a humoral and a cellular immune response. B cells that produce neutralising antibodies and cytotoxic T lymphocytes play an important role in these major host defence mechanisms. Furthermore, antigen-activated helper T cells stimulate antibody production by B cells. Recently, a synthetic peptide vaccine has been demonstrated to elicit an effective protective immune response.^{1,2} These synthetic peptides appear to have a similar structure to the B-cell and T-cell epitopes within the protein molecule responsible for the induction of the protective humoral response.³

Porphyromonas gingivalis has been strongly associated with the development of periodontal disease⁴ and fimbriae are considered to mediate its adherence to host cells.^{5,6} Fimbria-specific antibodies against *P. gingivalis* have been demonstrated in the serum of patients with adult periodontitis⁷ and antibody-secreting cells specific for fimbriae have been found in the inflamed gingival tissue of the same patients.⁸ Furthermore, *P. gingivalis* fimbriae are highly immunogenic and it has been shown that subcutaneous or oral immunisation with *P. gingivalis*

fimbriae in BALB/c mice or guinea-pigs induced marked humoral and cellular immune responses.^{9–11}

Recently, we demonstrated that *P. gingivalis* strain 381 fimbriae and related synthetic peptide segments exhibited multifunctional properties such as antigenicity, mitogenicity and polyclonal B cell activation (PBA) for mouse splenocytes. Chemotactic activity, induction of production of pro-inflammatory cytokines such as tumour necrosis factor- α , interleukin-1 (IL-1), IL-6 and IL-8 in human monocyte and macrophage cultures, and haemagglutinating activity were also recorded.^{12–14} The locations of the peptides that produced these immunobiological effects were scattered throughout the N-terminal, central and C-terminal regions of the fimbrial subunit protein (fimbriin). These results indicate that *P. gingivalis* fimbriae consist of a multifunctional protein.

In this study, 67 overlapping 10-mer synthetic peptides from the amino-acid sequence of *P. gingivalis* strain 381 fimbriin¹⁵ were synthesised by the methods of Geysen *et al.*¹⁶ and used to determine the immunodominant domain(s) by an enzyme-linked immunosorbent assay (ELISA) with serum specimens from patients with adult periodontitis. The region(s) that induced T-cell proliferation activity in mice immunised with the fimbriae were also examined. The study was also designed to determine whether *P. gingivalis* fimbriae or their synthetic peptide segments could

induce a protective immune response in guinea-pigs infected with *P. gingivalis*.

Materials and methods

Animals

Male albino guinea-pigs (300–350 g) of a closed colony were purchased from Nihon Rabbit Inc., Osaka, Japan. Six-week-old male BALB/c mice were obtained from Japan SLC Co., Shizuoka, Japan.

Adjuvants

Sodium β -*N*-acetylglucosaminyl-(1 $\rightarrow$ 4)-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-(L)-stearoyl-(D)-*meso*-2,6-diaminopimelic acid-(D)-amide-D-alanine (GM-53), a stearoyl derivative of the disaccharide tetrapeptide isolated from *Lactobacillus plantarum* cell wall,¹⁷ was generously supplied by Dainippon Pharmaceutical Co., Osaka, Japan. Freund's incomplete adjuvant (FIA) was purchased from Difco.

Bacteria and preparation of fimbriae from *P. gingivalis* strain 381

P. gingivalis strain 381 was grown anaerobically in GAM Broth (Nissui, Tokyo, Japan) supplemented with haemin 5 mg/L and menadione 10 mg/L, and fimbriae were prepared as described previously.⁹ The basic structure of the fimbriae (fimbriin) was identified as a single band of the 41-kDa subunit protein by SDS-PAGE.⁹

Peptide synthesis

Sixty-seven peptides representing the complete *P. gingivalis* fimbriin sequence (amino acid residues 1–337,¹⁵ as shown in fig. 1) were synthesised in duplicate, with a multi-pin peptide synthetic kit (Chiron Mimotopes Pty Ltd, Clayton, Victoria, Australia) (the first 66 were sequential overlapping peptides of 10 amino acids—10-mer overlapping by five amino acids—and the last peptide included seven amino acids). The peptides were synthesised on the tips of polyethylene pins by F-moc solid-phase synthesis as described by the manufacturer.¹⁶ The synthesised peptides were used to detect specific binding of antibodies by a modified ELISA as described below.

For T-cell epitope mapping, the 67 synthetic peptides (fig. 1), spanning the entire fimbriin sequence,¹⁵ were synthesised by a simultaneous multiple and cleavable peptide synthesis kit (Chiron Mimotopes Pty Ltd).¹⁸ Peptides were synthesised on polyethylene rods and cleaved from the rods in 96-well round-bottomed microtitration plates (Corning, New York, USA); these were diluted in RPMI 1640 (Biken, Osaka, Japan) for use in the T-cell proliferation assay as described below.

For guinea-pig immunisation, peptides were synthesised with a model 9050 peptide synthesiser (Japan Millipore Ltd, Tokyo, Japan) by the solid-phase methods.¹⁹ After cleavage of the protecting groups with trifluoroacetic acid:*m*-cresol:ethanedithiol:methanesulphonic acid (90:2.5:5:2.5), each peptide was purified by high performance liquid chromatography on a reversed phase column (Capcell Pak C18 SG120, 1.5 $\times$ 15 cm; Shiseido, Tokyo, Japan) 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 peptide was collected by monitoring the absorbance at 220 nm. The purified peptide 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).

ELISA for anti-peptide antibodies

The pins, carrying the peptides, were blocked with 200 μ l of phosphate-buffered saline (PBS), pH 7.5 containing bovine serum albumin (BSA) 1% and Tween 20 0.1% (PBS-BSA-T) for 1 h at room temperature. They were incubated overnight at 4°C in 200 μ l of serum, diluted 10⁴ in PBS-BSA-T with sodium azide 0.5%, obtained from a patient with adult periodontitis, with or without pre-absorption with *P. gingivalis* fimbriae. The fimbriae had been incubated with serum specimens (from a total of 12 patients) for 2 h at 37°C,¹² and washed in PBS, pH 7.5, containing Tween 20 0.1%. Antibody was detected with alkaline phosphatase-conjugated goat anti-human immunoglobulin G (IgG; Zymed Laboratories Inc., San Francisco, CA, USA) at a dilution of 10³, washed with PBS, pH 7.5, containing Tween 20 0.1%, then incubated with 100 μ l of *p*-nitrophenyl-phosphate (Phosphatase Substrate 104; Sigma) 1 mg/ml dissolved in diethanolamine solution, pH 9.8. The absorbance of the developed product was detected at 405 nm by a microplate reader and the mean absorbance value for each pair of pins was calculated. On completion of the ELISA, the pins were cleaned by sonication in disruption buffer—SDS 1%, 2-mercaptoethanol 0.1% in 0.1 M sodium dihydrogen orthophosphate, pH 7.2—at 60°C. After washing in distilled water (55°C) and immersion in a boiling methanol bath for 2 min, the pins were air-dried and then stored in sealed containers with silica gel at room temperature in preparation for the next assay.¹⁶ Specific binding of antibodies was calculated as follows: specific binding activity (ΔA_{405}) = absorbance at 405 nm (A_{405}) in the patient serum – A_{405} in the serum with pre-treatment of absorption with fimbrial protein.

T-cell proliferation assay

Male BALB/c mice (6-week-old) were immunised

Fig. 1. Predicted structures of *P. gingivalis* strain 381 fimbrial¹⁵ and schematic representation of the 67 overlapping synthetic peptides from the fimbrial protein with predicted T-cell epitopes according to α -helical periodicity and amphipathicity (A), Rothbard and Taylor motifs (R) and I-A^d (D) and I-E^d (d) binding motifs by TSites program.²⁶

subcutaneously with *P. gingivalis* fimbrial protein 100 μ g and the same dose of GM-53 as an adjuvant in FIA. Eight days after immunisation, a spleen cell suspension was applied to a nylon wool column (Wako Pure Chemical, Osaka, Japan)²⁰ and the eluted lymph-

oid cells (4×10^7) were added to the tissue-culture dish coated with goat anti-mouse immunoglobulin (Ig) (Zymed). After incubation for 2 h at 4°C to remove Ig-bearing cells, the T-cell enriched fraction (4×10^5 cells/well) along with thioglycollate-induced perito-

neal macrophages (2×10^5 /well) from BALB/c mice as antigen-presenting cells,²¹ suspended in RPMI 1640 (Biken) supplemented with 50 mM 2-mercaptoethanol and fetal bovine serum (FBS; HyClone, Logan, UT, USA) 10%, were cultured with various synthetic peptides (10 µg/well) in the wells of a 96-well flat-bottomed plate (Falcon 3072; Becton Dickinson Labware, Lincoln Park, NJ, USA). The plates were incubated at 37°C in an atmosphere of CO₂ 5% and air 95% for 3 days and were pulsed for a final 8 h with 37 KBq [³H]thymidine (specific activity 74 GBq/mmol; ICN Radiochemicals, Irvine, CA, USA). The cells were harvested and the radioactivity incorporated was determined by liquid scintillation counting. The T-cell stimulation index (SI) was calculated as follows: SI = cpm in cultures with test specimen/cpm in cultures without test specimen. Each experiment was performed at least three times.

Humoral immune response to P. gingivalis fimbriae and their synthetic peptides

Groups of six guinea-pigs were inoculated with the fimbriae (10–500 µg) or the synthetic peptide segments (100–500 µg) by injection into the left hind footpad, with or without 500 µg of adjuvant GM-53 in FIA on day 0. The levels of IgG antigen-specific antibodies in the serum were determined by ELISA¹¹ 27 days after immunisation. Values were expressed as mg or µg of IgG/ml of serum.

Protective effects of P. gingivalis fimbriae and their synthetic peptides on intracutaneous infection with P. gingivalis

P. gingivalis strain 381 was cultured in 500 ml of GAM broth at 37°C for 16–18 h until the optical density at 550 nm reached 1.3. After centrifugation, serial dilutions of the bacterial cell suspension were prepared and the cell count was determined with a Petroff-Hausser bacteria counter (Hausser Scientific Partnership, Horsham, PA, USA). Bacterial cells were washed with PBS, pH 7.4, and the numbers of cells were adjusted to a concentration of 1×10^{11} /ml with PBS. After shaving, 0.2 ml of the cell suspension (2×10^{10}) was injected intracutaneously into the right flank of each guinea-pig with a tuberculin syringe, 28 days after the immunisation described above. The area (longest length [mm] × shortest length [mm]) of redness in the lesion was determined 24 h after the inoculation of bacteria.

Computer predictions of antigenic determinants and statistics

The amino acids of the *P. gingivalis* fimbrilin sequence were analysed by prediction parameters such as hydrophilicity,²² chain flexibility,²³ and β -turn

probability²⁴ for possible antigenic determinants.²⁵ The T-cell epitopes were also predicted according to the TSites program²⁶ as shown in fig. 1.

Comparisons between groups were done by Student's *t* test for independent samples.

Results

Analysis of predictive T-cell epitopes of P. gingivalis fimbrilin

Prediction of T-cell epitopes for *P. gingivalis* fimbrilin with the TSites program²⁶ that combined four predictive algorithms (amphipathicity, Rothbard and Taylor motifs, and I-A^d and I-E^d binding motifs), demonstrated that among the peptides that stimulated T-cell proliferation, positions 36–45 present in peptide 8, 156–165 (peptide 32), 206–215 (peptide 42), 216–225 (peptide 44) and 236–245 (peptide 48) but not 41–50 (peptide 9) contained the predictive algorithms shown in fig. 1. Position 156–165 also coincided partially with I-A^d motifs. I-E^d and I-A^d motifs as well as Rothbard and Taylor motifs were located at position 121–130, but the corresponding peptide 25 did not induce T-cell proliferation (fig. 3).

Analysis of immunodominant epitopes of P. gingivalis fimbrilin

Fig. 2 shows antigen-specific binding of IgG (ΔA_{405}) in the representative serum specimens from three patients with adult periodontitis to 66 overlapping 10-mer synthetic peptides and the last peptide that included seven amino acids, covering the 337-amino-acid (AA) fimbrilin of *P. gingivalis*. The distinct IgG responses against peptides 6 and 7 (region I), 19 and 20 (region II), 24 and 25 (region III), 29 and 31 (region IV), 41 and 47 (region V), and 56 and 59 (region VI) were found in serum specimens from the patients. Six common immunodominant domains ($\Delta A_{405} \geq 0.2$) were identified: region I, located between AA 26 and 40; region II, between AA 91 and 105; region III, between AA 116 and 130; region IV, between AA 141 and 160; region V, between AA 201 and 240; and region VI, between AA 276 and 300.

Proliferative response of T cells from BALB/c mice immunised with P. gingivalis fimbriae

T cells from BALB/c mice immunised with *P. gingivalis* fimbriae induced an antigen-specific proliferative response to both the native fimbriae as well as six 10-mer synthetic peptides that correspond to the sequence of fimbrilin (fig. 3). Epitope mapping with synthetic peptides revealed that peptides 8 (AA 36–45), 9 (AA 41–50), 32 (AA 156–165), 42 (AA 206–215), 44 (AA 216–225) and 48 (AA 236–245) were definitely recognised (stimulation index of ≥ 2.0) by T cells of

Fig. 2. Specific binding of IgG (ΔA_{405}) in serum specimens from three representative patients with adult periodontitis to 67 overlapping synthetic peptides of the sequence between amino acids 1 and 337 of the fimbriin of *P. gingivalis* strain 381. Serum antigen-specific IgG levels were determined by ELISA. The binding assay was done in triplicate and the SEM did not exceed 10%. Regions I and VI indicate the common immunodominant domains ($\Delta A_{405} \geq 0.2$).

Fig. 3. The proliferative response of T cells from BALB/c mice immunised with *P. gingivalis* fimbriae after stimulation with 67 overlapping synthetic peptides of the sequence of fimbrialin from *P. gingivalis* strain 381. The experiments were performed at least three times and only representative data are indicated. Values are expressed as T-cell stimulation index and the SEM did not exceed 10% in any experiment.

BALB/c mice immunised with *P. gingivalis* fimbriae (fig. 3).

Induction of humoral immune response in guinea-pigs after immunisation with P. gingivalis fimbriae or their synthetic peptide segments

P. gingivalis strain 381 fimbriae (10–500 µg/animal) with or without 500 µg of the semi-synthetic adjuvant GM-53 in FIA were administered in the left hind footpad of guinea-pigs. Immunised animals developed significantly raised serum anti-fimbrial IgG antibody levels in a dose-dependent fashion (fig. 4). The production of fimbria-specific IgG antibody was maximal with an inoculum of 500 µg and reduced slightly with 1000 µg/animal (data not shown). The addition of adjuvant GM-53 in FIA produced increased levels of anti-fimbria IgG antibodies in serum (fig. 4).

Peptide segments that corresponded to three of the six regions identified as immunodominant epitopes of *P. gingivalis*—FP381(142–161), FP381(202–221) and FP381(216–243)—were selected and synthesised. The ability of these three synthetic peptides to induce IgG responses in guinea-pigs was examined. Subcutaneous injection of FP381(142–161), FP381(202–221) or FP381(216–243) with GM-53 in FIA produced higher levels of antigen-specific IgG than inoculation of the same antigens without GM-53 in FIA (fig. 4).

Development of lesions in guinea-pigs infected with P. gingivalis

Lesions induced by an inoculum of 2×10^8 cells/site were significantly larger than those of the control group treated with PBS alone ($p < 0.01$) (fig. 5). The lesions consisted of clearly demarcated areas of erythema at an inoculum size of 2×10^8 cells/site 16–24 h after the inoculation of *P. gingivalis* and their surface area was directly associated with inoculum size. Dermonecrotic lesions occurred with an inoculum of ($c. 2-5$) $\times 10^{10}$ cells/site 24 h after inoculation; the lesions drained externally through the overlying epidermis 2–3 days later. All the lesions healed within 12–14 days.

Protective immunisation with the synthetic peptides that mimic epitopes of P. gingivalis fimbriae

The protective effect against the development of *P. gingivalis*-infected lesions was assessed in guinea-pigs immunised with the native fimbria or synthetic peptide segment, with GM-53 in FIA or with FIA alone (control group) (table). Immunisation with *P. gingivalis* fimbriae and GM-53 in FIA resulted in 24% reduction in the size of infected lesions when compared with that of animals given FIA alone (control group). The effect of immunising guinea-pigs with

Fig. 4. The serum immune responses in guinea-pigs immunised subcutaneously with *P. gingivalis* fimbriae and three synthetic peptide segments: FP381(142-161), (202-221) and (216-243), with or without 500 µg of adjuvant GM-53 in FIA, or GM-53 in FIA or FIA alone. Serum IgG antibody levels were determined by ELISA 27 days after immunisation. Values (mean and SEM) are expressed as mg or µg of IgG/ml of serum. * Indicates a statistical difference ($p < 0.01$) compared with the FIA control.

Fig. 5. Mean lesion area in guinea-pigs inoculated with *P. gingivalis* strain 381 in PBS at various inoculum sizes (cells/site). The mean lesion area increased in proportion to the size of the inoculum. Values (mean and SEM) are expressed as the area of redness (mm²). * Indicates a statistical difference compared with the PBS control ($p < 0.01$).

Table. The protective immune responses of *P. gingivalis* fimbriae and their synthetic peptides to *P. gingivalis*-infected guinea-pigs

Immunisation*	Redness area	
	Mean (SEM) mm²	Inhibition (%)
<i>P. gingivalis</i> fimbriae with GM-53 in FIA	1486 (250)†	24
<i>P. gingivalis</i> fimbriae in FIA	1648 (113)†	15
FP381(142–161) with GM-53 in FIA	992 (122)‡	49
FP381(202–221) with GM-53 in FIA	240 (32)‡	88
FP381(216–243) with GM-53 in FIA	855 (75)‡	56
GM-53 in FIA	1800 (210)	8
FIA alone	1947 (107)	0

* Seven groups of six guinea-pigs were immunised by subcutaneous injection of 500 µg of test material with or without 500 µg of GM-53 in FIA or FIA alone (see *Methods*).

The statistical difference between the test and FIA control specimens was † $p < 0.05$ and ‡ $p < 0.01$, respectively.

FP381(142–161), FP381(202–221) or FP381(216–243) was a reduction in the size of lesions by 49%, 88% and 56%, respectively which were greater reductions than those recorded for the group immunised with fimbriae (table).

Discussion

P. gingivalis possesses various surface components such as fimbriae, capsules and lipopolysaccharide.^{27–31} The fimbriae from *P. gingivalis* are highly antigenic; anti-fimbrial antibodies have been detected in the serum of patients with adult periodontitis⁷ and an

increase in the number of *P. gingivalis* fimbria-specific antibody-secreting cells in the inflamed gingival tissues of the same patients has been described.⁸ Furthermore, elevated anti-fimbrial antibody levels in serum and saliva as well as increased fimbria-specific antibody-secreting cells in various tissues have been demonstrated in BALB/c mice immunised subcutaneously or orally with *P. gingivalis* fimbriae in FIA or liposomes.^{9, 10, 32} More recently, we have demonstrated that *P. gingivalis* fimbriae induced humoral as well as cellular immune responses in guinea-pigs and BALB/c mice but not in BALB/c *nu/nu* mice.¹¹

Six regions with immunodominant epitopes for *P. gingivalis* strain 381 fimbriin were detected in serum specimens from patients with adult periodontitis (fig. 2). Immunodominant regions IV and V identified by serology corresponded to computer predictions for moderate or high hydrophilicity,²² chain flexibility,²³ and β -turn probability,²⁴ indicating a possible surface-exposed site on the native fimbrial protein. On the basis of the higher hydrophilicity and β -turn probability found in region VI, compared to these features in regions IV and V, the immunodominant epitope is likely to be in region VI. However, the corresponding peptide did not always react specifically with patient serum specimens and, therefore, was not used in later experiments (fig. 2). Regions I and II indicated only high chain flexibility or hydrophilicity.

In previous studies, BALB/c and DBA/2 mice (H-2^d) were high responders, CBA/J and C3H mice (H-2^k) intermediate, and C57BL/6 mice (H-2^b) low responders to *P. gingivalis* fimbriae.³³ Furthermore, in H-2 recombinants on B10 background, B10.D2 (H-2^d) followed by B10.BR (H-2^k) were high responders, whereas C57BL/10 (B10, H-2^b) were low responders to the fimbrial antigen. These results suggested strongly that the antibody responses to the fimbrial antigen were restricted by the H-2 haplotype. In this study, six synthetic peptides induced T-cell proliferation in BALB/c mice (H-2^d) immunised with the fimbriae in positions AA 36–45 present in peptide 8, 41–50 in peptide 9, 156–165 in peptide 32, 206–215 in peptide 42, 216–225 in peptide 44 and 236–245 in peptide 48 (fig. 3). Among these synthetic peptides, Asn (N) was commonly observed in the central region of sequences 156–165 (AKKQSNLFGA), 216–225 (ENDYSANGGT) and 236–245 (KLQKNGADLA) and Gly-Ala (GA) or Gly-Gly (GG) was also seen in the C-terminal region of the amino acid sequences (fig. 1). It appears that these amino-acid residues may play an important role as a T-cell epitope. Further investigations, including the establishment of the fimbria-specific T-cell clones in adult periodontitis patients and in animals immunised with *P. gingivalis* fimbriae, and the analysis of T-cell epitopes, are being conducted.

The three synthetic peptide segments, FP381(142–161), (202–221) and (216–243), that correspond to regions IV and V of the amino-acid sequences of the fimbriin immunodominant epitopes

as well as the T-cell epitopes, produced a protective immune response in guinea-pigs challenged with *P. gingivalis* (table). This effect was maximal with FP381(202–221) and all the synthetic peptides produced a stronger protective effect than that induced by native fimbriae. It is interesting that the combination of fimbrial antigen and adjuvant GM-53 resulted in a superior effect when compared with fimbrial antigen alone (table). Recently, we reported that subcutaneous injection of *P. gingivalis* fimbriae and FP381(202–221) with adjuvant GM-53 in FIA markedly induced humoral as well as cellular immune responses, and its antigenicity is suggested to be independent on thymocytes in BALB/c mice.¹¹ However, subcutaneous administration of FP381(61–80), which corresponds to peptides 13 and 15 (fig. 1), indicating very weak immune response with patient

serum specimens (fig. 2) and the T-cell proliferative response (fig. 3), induced lesser degrees of humoral and cellular immune responses in guinea-pigs than did FP381 (202–221).¹¹ The latter peptide also induced a weaker protective immune response in *P. gingivalis*-challenged guinea-pigs (unpublished results). The immunodominant peptide sequences may induce T-cell proliferative responses (figs 2 and 3) but this area requires further study. Synthetic vaccines of the future against infectious diseases, including periodontitis, will probably consist of a B-cell epitope region mixed with or coupled to a T-cell epitope region within an appropriate protein molecule as an antigen, together with a powerful and non-toxic adjuvant. The results of the present study suggest that the development of a vaccine of this type for periodontal disease caused by *P. gingivalis* is possible.

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