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Characterization of T Cell Responses to the RgpA-Kgp Proteinase-Adhesin Complexes of Porphyromonas gingivalis in BALB/c Mice


Porphyromonas gingivalis is a Gram-negative bacterium strongly associated with chronic periodontitis, an inflammatory oral disease. A major virulence factor common to all characterized strains of P. gingivalis is the RgpA-Kgp proteinase-adhesin complexes. In this study, we investigated T cell proliferative and cytokine responses to the RgpA-Kgp complexes and identified T cell epitopes in BALB/c mice utilizing Pepscan methodology. T cell proliferative responses were found to be predominantly directed toward the proteinase catalytic domains. Eleven T cell epitopes were identified using RgpA-Kgp-primed lymph node T cells (IL-4 dominant) and 21 using an RgpA-Kgp-specific T cell line (IFN-γ dominant), with 5 T cell epitopes, including the immunodominant epitope peptide 22, common to both T cell populations. Peptide 22 (AS9ANYTAHGSTAWADP453) from the Kgp proteinase catalytic domain induced a Th2 cytokine response in mice, and peptide 22-primed T cells had a Th2 cytokine profile when stimulated with the RgpA-Kgp complexes. Truncation and alanine scanning of peptide 22 identified the minimum epitope (AS9TAHGSETAWA461), and residues His444, Glu447, and Trp450 as critical for T cell proliferation. With a view to vaccine development, peptide 22 was incorporated into a synthetic peptide polymer. Peptide 22 polymer induced strong T cell proliferation and crossreactivity to native RgpA-Kgp complexes. In conclusion, we have identified a major T cell epitope of P. gingivalis and established that antigenicity of the T cell epitope is retained when delivered as a peptide polymer. The strategies employed here may have potential in the development of a synthetic peptide vaccine for P. gingivalis. The Journal of Immunology, 2008, 181: 4150–4158.

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The RgpA-Kgp complexes have been identified as a major virulence factor contributing to the pathogenicity of *P. gingivalis*, as mutant strains of the bacterium that lack the proteinases and adhesins are less virulent in the mouse lesion and in periodontitis models (22, 23). Additionally, when a preparation of the RgpA-Kgp complexes was used as a vaccine, it was found to give protection in the mouse lesion and periodontitis models and in the rat periodontitis model (8, 9, 24). A number of peptides corresponding to both the proteinases and adhesins when conjugated to diphtheria toxoid and used as immunogens were protective in the mouse lesion and in periodontitis models (8, 24). Studies have also shown that protection in mice is associated with the production of a *P. gingivalis* Ag-specific IgG1 subclass and Th2 IL-4 cytokine response, whereas disease was associated with an IgG3 Ab response and a predominant IFN-γ (Th1) cytokine response (8, 25).

While the exact role of T cells in periodontitis has yet to be fully elucidated, the cells have been reported to be present in the inflammatory infiltrate during periodontal disease (26). Furthermore, studies by Baker et al. (27, 28) have suggested that CD4+ T cells, and not CD8+ T cells, are associated with bone resorption after oral infection with *P. gingivalis* in mice. Other reports have indicated that the presence of proinflammatory cytokines IFN-γ and IL-6 produced by CD4+ T cells are also associated with bone loss (27) and that IL-10 (a Th2-type cytokine) knock-out mice exhibited more *P. gingivalis*-induced alveolar bone loss than that of wild-type mice (29). T cells have also been reported to play a role in protection against bone loss in the mouse periodontitis model, with IL-4 being the predominant cytokine secreted by these protective cells (8). Furthermore, a bias toward a Th1 response resulted in elevated levels of periodontal tissue inflammation and alveolar bone loss in mice after challenge with *P. gingivalis*, whereas mice that were biased toward a Th2 response did not develop periodontal bone loss (30).

T cells are therefore likely to play an important role in chronic periodontitis. However, the T cell responses to the RgpA-Kgp complexes of *P. gingivalis* have not yet been determined. In this study, we identify the major T cell stimulatory domains of the RgpA-Kgp complexes, identify the predominant T cell epitopes, and characterize the T cell cytokine responses to the RgpA-Kgp complexes. Furthermore, we define the immunodominant epitope utilizing truncation and alanine scanning techniques and evaluate the epitope immunogenicity in a peptide polymer vaccine construct.

### Materials and Methods

#### Growth of *P. gingivalis* and purification of RgpA-Kgp, RgpA, and recombinant KgpΔ1

*P. gingivalis* strain W50 (31) and *P. gingivalis* mutant strains RgpA- (W501) (32) and KgpΔ1 (K1A) (33) were grown as previously described (34). *P. gingivalis* RgpA-Kgp, RgpA, and Kgp were prepared and characterized as previously described (34). Recombinant KgpΔ1 was produced as previously described by Frazer et al. (35).

#### Immunization protocols

BALB/c mice were obtained from the animal facility at the Department of Immunology and Microbiology at The University of Melbourne, and animal experimentation was approved by the University of Melbourne animal ethics committee. RgpA-Kgp complexes (50 μg/mouse) or peptide 22 (30 nmol/mouse) was emulsified in CFA (Sigma-Aldrich) and used to immunize BALB/c mice subcutaneously in the hind leg.

#### T cell proliferation assays

Lymphocytes were prepared from pooled inguinal and popliteal lymph node cell suspensions of BALB/c mice primed 7 days previously with the RgpA-Kgp complexes. Splenes were isolated from nonimmunized mice as a source of syngeneic APCs. Lymph nodes and spleens were collected in enriched DMEM/Ham’s nutrient mixture F-12 (DMEM/F-12) supplemented with 10% (v/v) heat-inactivated (56°C, 30 min) FBS, 2 mM glutamine, 2 mM sodium pyruvate, 0.1 mM 2-ME, 30 μg/ml gentamicin, 100 IU/ml penicillin, and 100 μg/ml streptomycin (JRH Biosciences), 1-arginine (116 mg/ml), 1-asparagine (36 mg/ml), and folic acid (6 mg/ml, Sigma-Aldrich), and single-cell suspensions were made by passing the lymph nodes or spleens through a wire mesh. RBCs were removed from the spleen cell suspension by treatment with ammonium Tris buffer (17 mM Tris-HCl, 140 mM ammonium chloride in Milli-Q water (pH 7.2)) for 5 min at room temperature, and then washed three times in enriched DMEM/F-12 (5 min, 300 × g, EconoSpin, Sorvall Instruments, DuPont).

Monocytes and dead cells were removed from the lymph node cell suspension using Lympholyte-M (Cedarlane Laboratories) as per the manufacturer’s instructions. Lymph node T cells were cultured using the mouse T cell recovery columns (Cedarlane Laboratories) as per the manufacturer’s instructions. T cells were cultured in enriched DMEM/F-12 at a concentration of 3 × 10^7 lymph node T cells/well in a 96-well microtiter plate (Nunc) in the presence of syngeneic γ-irradiated (2200 rads) spleen cells (3 × 10^5 cells/well) together with Ag (RgpA-Kgp, RgpA, and Kgp, recombinant KgpΔ1 or peptide) in a total volume of 250 μl. T cells were incubated for four days at 37°C in an atmosphere of 5% CO2 in air. One μCi [3H]thymidine (Amersham Biosciences) was added per well and incubated for a further 18 h. Cells were then lysed with cell lysis buffer (30 mM Tris-HCl, 100 mM EDTA, 1% N-lauroylsarcosine (pH 8)) and harvested onto glass-fiber filters using a Tomtec Harvester96 Mach III cell harvester. The glass-fiber filters were then air dried and sealed in plastic bags containing 5 ml of Betaplate Scint (PerkinElmer), and incorporation of [3H]thymidine was measured using a Wallac MicroBeta TriLux liquid scintillation counter (PerkinElmer). Data are expressed as stimulation index (S.I.)^3 = SD, where S.I. is the cpm divided by the negative control (no Ag) cpm.

#### Generation of an RgpA-Kgp-specific T cell line

Lymphocytes were prepared from pooled inguinal and popliteal lymph node cell suspensions from BALB/c mice primed 7 days previously with the RgpA-Kgp complexes and spleens were isolated from nonimmunized mice as a source of syngeneic APCs. Isolated T cells were resuspended in enriched DMEM/F-12 at a concentration of 2 × 10^7 T cells/ml and incubated with syngeneic γ-irradiated (2200 rads) spleen cells (2 × 10^5 cells/ml), and 1 μg/ml RgpA-Kgp Ag for 5 days s. The T cells were subsequently expanded using 25 U/ml of human recombinant IL-2 (rhIL-2, Sigma-Aldrich) and enriched DMEM/F-12 for a further 5 days before being isolated with Lympholyte-M. The cycle of Ag stimulation and rhIL-2 expansion was then repeated to maintain the RgpA-Kgp-specific T cell line.

#### ELISPOT assay

ELISPOT assay was performed as previously described (8) using the RgpA-Kgp complexes or peptide 22 as the stimulating Ag.

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3 Abbreviations used in this paper: S.I., stimulatory index; rhIL-2, recombinant human IL-2.
Peptide synthesis and purification

Unless otherwise stated, chemicals were of peptide synthesis grade or its equivalent. O-benzotriazole-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HOBt), diisopropylethylamine (DIPEA), N,N-dimethylformamide (DMF), piperidine, trifluoroacetic acid (TFA), and 9-fluorenylmethoxycarbonyl (Fmoc) protected amino acids were obtained from Auspep.

Overlapping, 15-mer peptides (offset by 10 residues and overlapping by 5 residues) representing the sequence of the RgpA-Kgp from *P. gingivalis* (strain W50) were manually synthesized on diketopiperazine pins (Mimotopes) using standard solid-phase peptide synthesis protocols for Fmoc chemistry. The Fmoc group was removed with 20% (v/v) piperidine in DMF (20 min). After washing (DMF 2 min, methanol 4 × 2 min), the pins were air dried (40 min). Acylation was accomplished with HBTU ((O-(1H-benzotriazole-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate)/HOBt) activation with 4 equivalents of Fmoc-amino acid and 6 equivalents of DIPEA with respect to pin substitution for 4 h, and was followed by a further acylation (double coupling) for 18 h. Before cleavage from the pins, the side-chain protecting groups were removed by submerging the pins in TFA/ethanedithiol/anisole (95:2.5:2.5) for 2.5 h at room temperature with gentle agitation. After removal of the side-chain protecting groups, the pins were washed: methanol (10 min), 0.5% (v/v) acetic acid in methanol/water (1:1 (v/v), 1 h), and then Milli-Q water (5 min). Cleavage of the peptides from the pins was achieved by submerging the pins in 0.1 M ammonium bicarbonate (pH 8.4) containing 40% (v/v) acetonitrile for 30 min with sonication. Cleaved peptides were lyophilized and stored at −20°C.

Peptide 22 (439ANYTAHGSETAWADP453) was synthesized on Fmoc-PAL-PEG-PS resin (PerSeptive Biosystems) using an AB 431A peptide synthesizer (Applied Biosystems) and standard solid-phase synthesis protocols for Fmoc chemistry.

Acryloyl-amino hexanoic acid (Ahx) peptide 22 used for polymerization was synthesized and purified by reversed-phase HPLC as previously described (8, 36). The purified peptides were analyzed by mass spectrometry, using a Voyager DE MALDI-TOF mass spectrometer (PerSeptive Biosystems) and had the observed masses of 1589.76 Da for peptide 22 (calculated mass of 1589.65 Da), and 1757.23 Da for acryloyl-Ahx-peptide 22 (calculated mass of 1756.86 Da).

Polymerization of acryloyl peptides

Polymerization of acryloyl peptide 22 monomer (acryloyl-Ahx-peptide 22) was achieved by the addition of 50-fold molar excess of acrylamide (with respect to the amount of acryloylated peptide) and was initiated by the addition of ammonium persulfate and N,N,N′,N′-tetramethylethylenediamine (TEMED). The reaction was conducted in 6 M guanidine-HCl containing 2 mM EDTA and 0.5 M Tris (pH 8.3) and was left at room temperature for 18 h under nitrogen. Polymer was purified using size-exclusion chromatography on a Superose 12 column 10/300 GL (10 × 300 mm) installed on a Waters Delta 600/Millenium HPLC system (Waters). The chromatogram was developed at a flow rate of 0.5 ml/min using 50 mM ammonium bicarbonate and monitored at 280 nm. Material eluted in the void volume from the column was collected.

*Statistical analysis*

The alanine scan data were found to be not normally distributed using Levene’s test of homogeneity of variances (SPSS for Windows, release 6.0; SPSS); hence, the S.I. for the alanine scan was statistically analyzed using the Kruskal-Wallis test and the Mann-Whitney U Wilcoxon rank sum test with a Bonferroni correction for type 1 error (SPSS for Windows, release 6.0; SPSS) (37). The S.I. for the epitope mapping and...
A and used to stimulate RgpA-Kgp-primed lymph node T cells (Fig. 3). The 192 15-mer overlapping peptides representing the unique sequences of RgpA and Kgp were pooled into groups of 20 peptides and used to stimulate RgpA-Kgp-primed lymph node T cells (A). The groups of 20 peptides that were found to induce T cell proliferation were further divided into groups of 5 peptides and used to stimulate RgpA-Kgp-primed lymph node T cells (B). The groups of 5 peptides that were found to induce T cell proliferation of >2 S.I. units were further divided into single peptide groups and used to stimulate RgpA-Kgp-primed lymph node T cells (C).

**Results**

The antigenicity of the different components of the RgpA-Kgp complexes were investigated by stimulating RgpA-Kgp-primed T cells with a range of concentrations of RgpA-Kgp, RgpA, Kgp, and recombinant KgpA1 adhesin (rKgpA1) (Fig. 2). All the Ags that were tested stimulated T cell proliferation of RgpA-Kgp-primed T cells, but each Ag induced maximum T cell proliferation at different Ag concentrations. The Kgp Ag induced maximum proliferation at an Ag concentration of 0.78 pmol/ml, whereas the RgpA and RgpA-Kgp Ags induced maximum proliferation at concentrations of 1.56 and 6.25 pmol/ml, respectively. Recombinant KgpA1 induced maximum proliferation at the highest antigenic concentration of 200 pmol/ml. At an antigenic concentration of 0.78 pmol/ml, there was no significant difference in the proliferation of the RgpA-Kgp-primed T cells in response to the RgpA, Kgp, and RgpA-Kgp complexes; however, at that concentration, rKgpA1 induced significantly (p < 0.05) lower levels of T cell proliferation than did the proteinase Ags. The RgpA-Kgp complexes induced a higher maximum response than that of either RgpA or Kgp, and there was no significant difference in the maximum response induced by RgpA or Kgp.

**Cytokine and proliferative responses of RgpA-Kgp-primed lymph node T cells and a RgpA-Kgp-specific T cell line**

To establish an RgpA-Kgp-specific T cell line, lymph node T cells previously primed with the RgpA-Kgp complexes were cultured with the RgpA-Kgp complexes for 5 days and then expanded with rhIL-2 for a further 5 days. This cycle was repeated to establish a RgpA-Kgp-specific T cell line. The proliferative and cytokine responses induced by the RgpA-Kgp-specific T cell line and RgpA-Kgp-primed lymph node T cells were evaluated and compared (Fig. 3). The proliferative response of the T cell line when stimulated with the RgpA-Kgp Ag at 25 pmol/ml was twice as strong as the proliferative response of the RgpA-Kgp-primed lymph node T cells. The maximum T cell proliferation was observed at similar Ag concentrations (25 pmol/ml) for both cell types (Fig. 3A).

The cytokine secretion profile of the RgpA-Kgp-primed lymph node T cells and the RgpA-Kgp-specific T cell line is shown in Fig. 3, B and C. Stimulation of the lymph node T cells with the RgpA-Kgp complexes induced a predominant IL-4 cytokine response (Fig. 3B), where the number of cells producing IL-4 was significantly higher (p < 0.05) than the number of cells producing IFN-γ. Stimulation of the RgpA-Kgp-specific T cell line induced a predominant IFN-γ response (Fig. 3C), where the number of cells producing IFN-γ was significantly higher (p < 0.01) than the number of cells producing the IL-4 cytokine.

The ELISPOT data were analyzed for significance utilizing a one-way classification ANOVA with a posthoc Dunnett’s T3 test. Effect sizes, represented as Cohen’s (38) d, were calculated using the effect size calculator provided online by Evidence-Based Education U.K. website (http://cem.dur.ac.uk/ebuek/researc/effectsize/). According to Cohen (38), a small effect size is d = ±0.2 and <0.5, a moderate effect size is d = ±0.5 and <0.8, and a large effect size is d = ±0.8.
Identification of RgpA-Kgp T cell epitopes

As the cytokine profiles of the RgpA-Kgp-primed lymph node T cells and the RgpA-Kgp-specific T cell line were different, both T cell populations were used to identify RgpA-Kgp T cell epitopes. Peptide pools corresponding to Kgpcat (peptide groups 21–40) stimulated the strongest proliferation, followed by peptide pools corresponding to KgpA1 (groups 41–60 and 61–80) and RgpAcat (group 81–100). The cytokine profiles of the peptide 22-primed and Kgpcat-primed lymph node T cells are summarized in Table I.

The 192 individual peptides were also used to stimulate the RgpA-Kgp-specific T cell line. Twenty-one RgpA-Kgp T cell epitopes were identified (Fig. 4D). Seventeen of these epitopes were located within Kgpcat, two epitopes were located within KgpA1, and one epitope was located in each of the KgpA2/RgpA2 and RgpAcat domains. The sequences of these epitopes are shown in Table I. Peptide 15, 17, 22, 39, and 43 located in Kgpcat domain stimulated the strongest T cell proliferative response in the RgpA-Kgp-specific T cell line. Five of the identified epitopes (peptides 22, 23, 39, 16, and 17) were identified in both the RgpA-Kgp-primed lymph node T cells and RgpA-Kgp-specific T cell line (Table I). Peptide 22 (ANYTAHGSETAWADP) was found to induce a consistently stronger T cell proliferative response compared with the other epitopes.

Characterization of the T cell responses induced by the Kgpcat sequence ANYTAHGSETAWADP (peptide 22)

Peptide 22 was synthesized using standard solid-phase synthesis protocols for Fmoc chemistry, purified using reversed-phase HPLC purification and subsequently utilized in T cell proliferation and ELISPOT assays. Peptide 22 induced maximum T cell proliferation at an Ag concentration of 12.5 pmol/ml, which was approximately 4-fold higher than the RgpA-Kgp concentration required to maximally stimulate RgpA-Kgp-primed T cells (Fig. 5C). Maximum proliferation induced by the RgpA-Kgp complexes in peptide 22-primed lymph node T cells was at an Ag concentration of 12.5 pmol/ml, which was approximately 4-fold higher than the RgpA-Kgp concentration required to maximally stimulate RgpA-Kgp-primed T cells (Fig. 5C).

The cytokine secretion profiles of the peptide 22-primed and RgpA-Kgp-primed T cells when stimulated with peptide 22 were also investigated (Fig. 6). RgpA-Kgp-primed T cells had a higher

### Table I. Sequences of the peptides that stimulated proliferation of RgpA-Kgp-specific T cells

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<th>Origin</th>
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* Epitopes that were identified from both the RgpA-Kgp-primed lymph node T cells and the RgpA-Kgp-specific T cell line.
IL-4 response in comparison with IFN-γ secretion when stimulated with RgpA-Kgp complexes (p < 0.05, average effect size d = 21.87) or peptide 22 (average effect size d = 4.33) (Fig. 6). Peptide 22-primed T cells had a higher IL-4 response when stimulated with RgpA-Kgp complexes (average effect size d = 3.82); however, the differences in IL-4 and IFN-γ secretion induced by peptide 22 (average effect size d = 0.67) were not significant (Fig. 6).

Identification of the critical residues and minimal T cell epitope of peptide 22 (ANYTAHGSETAWDP)

To identify the minimum T cell epitope of peptide 22, 15 15-mer peptides overlapping by 14 residues and offset by 1 residue from residues Ala432 to Leu460 (Table II A) were used to stimulate peptide 22-primed lymph node T cells (Fig. 7A). The minimum epitope for peptide 22 was the inner sequence TAHGSETAWA, where the removal of the N-terminal Thr and C-terminal Ala both resulted in no T cell proliferation of the peptide 22-primed T cells. Peptide 9 (NYTAHGSETAWDP), which has the N-terminal Ala of peptide 22 deleted, was found to induce a significantly (p < 0.05) stronger T cell proliferative response than did the original sequence.

The alanine scanning technique was used to investigate which residues in peptide 22 are important for T cell proliferation (Fig. 7B). Twelve peptides (Table II B) were used to stimulate peptide 22-primed T cells and the proliferative responses were compared

<table>
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FIGURE 5. Peptide 22 stimulation of RgpA-Kgp-primed lymph node T cells (○) (A) and peptide 22-primed lymph node T cells (■) (B) (●) represents unstimulated cells). RgpA-Kgp Ag stimulation of RgpA-Kgp-primed lymph node T cells (●) and peptide 22-primed lymph node T cells (■) (C). Proliferation was detected using [3H]thymidine incorporation and is expressed as the S.I. Means are the average of triplicate assays ± SD.

FIGURE 6. Cytokine profile of RgpA-Kgp-primed or peptide 22-primed lymph node T cells stimulated with RgpA-Kgp complexes or peptide 22. Lymph node T cells were stimulated with 10 μg/ml of RgpA-Kgp complex or 50 nmol/ml peptide 22 for 48 h; IL-4 stimulation is shown by the open bars and IFN-γ stimulation is shown by the filled bars. Results are expressed as spot-forming cells per million and are the average of triplicate assays ± SD.

Table II. Sequences of the peptides used in the identification of the minimum epitope (A) and alanine screen (B) of peptide 22
The substitution of the His^{444}, Glu^{447}, and Trp^{450} residues with Ala resulted in no T cell proliferation of the peptide 22-primed T cells. Substitution of Thr^{442} and Gly^{445} and Pro^{453} residues with Ala all had a similar effect at significantly (*p < 0.05) reducing T cell proliferation (average effect size d = 2.77).

Characterization of the T cell proliferative response induced by peptide 22 polymer and crossreactivity of the peptide polymer to the native Ag

A peptide polymer strategy (36) that may have utility in the production of a multivalent peptide vaccine is the acryloylation and subsequent polymerization of synthetic peptides. To assess this strategy with \textit{P. gingivalis} epitopes, peptide 22 was acryloylated and polymerized, and the ability of the peptide 22 polymer to induce a T cell response was evaluated and compared with that of peptide 22 monomer (Fig. 8A). Lymph node T cells that were primed with peptide 22 were stimulated with both peptide 22 and peptide 22 polymer (Fig. 8A). Both peptide 22 monomer and peptide 22 polymer induced significant T cell proliferation. Maximum proliferation for both peptide 22 monomer and peptide 22 polymer was induced at 12.5 nmol/ml of Ag. The peptide 22 polymer was also tested for its crossreactivity with the native RgpA-Kgp complexes. Peptide 22 polymer-primed lymph node T cells were stimulated with the RgpA-Kgp complexes and the resultant proliferation is shown in Fig. 8B. The RgpA-Kgp complexes stimulated peptide 22 polymer-primed T cells with a maximum proliferation at 50 pmol/ml of the RgpA-Kgp complexes.

Discussion

In this study, we investigated the T cell response and identified T cell epitopes to different components of the RgpA-Kgp complexes using RgpA-Kgp-primed T cells and a RgpA-Kgp-specific T cell line. The RgpA-Kgp Ag induced a strong T cell proliferative response and a predominant IL-4 cytokine response from the RgpA-Kgp-primed lymph node T cells. Using these cells, the abilities of the different components of the RgpA-Kgp complexes were evaluated and the responses compared.

The RgpA-Kgp complexes, as well as the major components RgpA and Kgp, induced significant T cell proliferation at low Ag concentrations. However, stimulation of the RgpA-Kgp-primed T cells with high concentrations of RgpA, Kgp, and RgpA-Kgp resulted in reduced levels of T cell proliferation. This phenomenon may be explained by overstimulation with Ag leading to apoptosis, which has been reported in previous studies (39), but this effect may also be enhanced by the RgpA and Kgp proteinases, as these enzymes at high concentrations have been reported to induce apoptosis in other cell types (40–44). The substantially higher maximum T cell proliferative response induced by Kgp, RgpA-Kgp, and RgpA at low Ag concentrations compared with the adhesin domain rKgpA1 is consistent with the immunodominant T cell epitopes being localized in the proteinase catalytic domains.

To aid in T cell epitope identification, an RgpA-Kgp-specific T cell line was generated and was found to produce significantly higher levels of IFN-\(\gamma\) in comparison with IL-4 after stimulation.
with the RgpA-Kgp complexes, which was in contrast to that produced by RgpA-Kgp-primed lymph node T cells. The strong IFN-γ response induced by the T cell line indicates that the T cell line is of a Th1 cytokine-secreting phenotype. This phenotype may be attributable to the continual stimulation with the RgpA-Kgp complexes, which contain immunogenic LPS-like carbohydrates (45) that have been reported to be crossreactive with P. gingivalis LPS (19, 46). Previous work has reported that stimulation of T cells with LPS results in the production of local inflammation and Th1 immunoreactivity, including the production of a predominant IFN-γ cytokine response (47, 48).

T cell epitopes of the RgpA-Kgp complexes were identified utilizing RgpA-Kgp-primed lymph node T cells and the RgpA-Kgp-specific T cell line. Eleven T cell epitopes were identified using lymph node T cells and 21 T cell epitopes were identified using the T cell line. Five common T cell epitopes of the RgpA-Kgp-primed lymph node T cells and the RgpA-Kgp complex T cell line were identified. The Th1 cytokine phenotype nature of the RgpA-Kgp-specific T cell line shown by the predominance of IFN-γ production may have resulted in the different epitopes that were identified. Previous studies using wild-type mice and mice genetically deficient in IL-4 (IL-4−/−) or IL-12 (IL-12−/−) have reported that Th1 and Th2 cells can recognize unique epitopes, as well as some common T cell epitopes (49). Taken together, this suggests that most T cell epitopes identified using the RgpA-Kgp-specific T cell line may be Th1 T cell epitopes.

A considerably higher proportion of T cell epitopes were identified from the proteinase catalytic domains, with 81% of the epitopes having been identified from the Kgpcat proteinase domain alone. The strong T cell epitopes to the RgpA-Kgp complexes were identified utilizing RgpA-Kgp-primed lymph node T cells appear to be localized around the region of the active sites of the Kgpcat and RgpAcat proteinases, where the two strongest peptides (peptides 22 and 158) contain the catalytic histidine and cysteine residues, respectively, that form part of the proteinase active site of the Kgp and RgpA/B proteinases, respectively (50). These data indicate that the T cell proliferative responses to the RgpA-Kgp complexes are primarily associated with the proteinases.

The initial Pepscan screening identified peptide 22 as the immunodominant peptide; however, further analysis revealed that the most effective epitope at inducing T cell proliferation was peptide 22 minus the N-terminal alanine (NYTAHGSETAWADP). This indicates that the identification of the actual immunodominant T cell epitope within a given sequence requires at least two Pepscan studies: the initial Pepscan for the identification of the T cell stimulatory sequence, and the latter to define the immunodominant T cell epitope. The immunodominant T cell epitope was further defined by identifying the critical residues important for T cell stimulation by replacing each residue with alanine and analyzing their ability to induce T cell proliferation. In these experiments, three residues (His444, Glu447, and Trp450) were found to be critical for T cell proliferation, and these residues (Thr442, Gly445, and Pro453) when changed to Ala significantly reduced T cell proliferation.

A number of studies have reported that a protective immune response against P. gingivalis-induced lesions and bone loss is characterized by a specific antiinflammatory Ab isotype response and that this is associated with a P. gingivalis-specific predominant Th2 (IL-4) cytokine response (8, 9, 25). In contrast, P. gingivalis-induced bone loss in animal models and chronic periodontitis in humans have been characterized by a proinflammatory Ab response and a Th1 (IFN-γ) cytokine response (8, 25, 30). For an effective vaccine, it is important that the vaccine induces the desired Th cytokine response for protective immunity, and that this is perpetuated by the native Ag. Peptide 22 was found to induce a Th2 (IL-4) cytokine response, but, more importantly, peptide 22-primed T cells were able to be stimulated by native Ag to produce a Th2 (IL-4) response. We have previously identified and characterized PAS1K as a protective B cell epitope against P. gingivalis-induced lesion formation and periodontal bone loss when the peptide was conjugated with diphtheria toxoid and used as a vaccine in mice (8, 24). PAS1K is a 21-residue peptide that contains the peptide 22 sequence. PAS1K when conjugated to diphtheria toxoid was as effective at providing protection as the native RgpA-Kgp Ag against P. gingivalis (8, 24). Thus, the PAS1K sequence contains both Ag-specific B cell and T cell epitopes, and with the additional T cell help provided by diphtheria toxoid, may explain why this peptide was so effective at inducing protection.

Although peptides can be straightforwardly synthesized and purified, a number of problems arise when peptides alone are used as immunogens. Synthetic peptides alone are small and poorly immunogenic, and thus they require adjuvants or conjugation to a larger carrier protein. However, with peptide-protein conjugates, there is the problem of carrier-induced epitope-specific suppression, which reduces the Ab response to the peptide of interest (51). A way in which to circumvent the problems associated with peptides is by increasing the size and copy number of peptides using a peptide polymer strategy (36). Using that strategy, the peptide 22 polymer prepared induced a T cell proliferative response that was crossreactive with the native RgpA-Kgp Ag. While the T cell response to the peptide polymer was weaker than to the peptide monomer, the immunogenicity of polymer could possibly be enhanced by the incorporation of a spacer between the peptide and polymer backbone. The use of other polymer strategies such as poly(L-lactic acid) (PLA) and poly(L,L-lactic/glycolic acid) (PLGA) for the incorporation of peptides and proteins has been reported to successfully enhance the immunogenicity of peptide Ags and to induce the production of Ag-specific Abs (52–54). The multivalent polymer strategy is therefore an effective way for the incorporation of epitopes into a single synthetic construct that still retains peptide antigenicity, where incorporation of multiple epitopes into polymers may potentially overcome the major hurdle of MHC restriction.

In conclusion, the T cell responses to the RgpA-Kgp complexes were predominantly directed to the proteinase catalytic domains. Furthermore, an immunodominant epitope was located within the Kgp proteinase catalytic domain and induced a Th2 cytokine response. The epitope as a synthetic peptide was incorporated into a polymer as an effective immunogen. This polymer approach, where multiple epitopes could be incorporated into a multivalent construct, may have potential in the development of a synthetic vaccine against P. gingivalis.

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Disclosures
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