Construction and immunologic evaluation of a *Porphyromonas gingivalis* subsequence peptide fused to hepatitis B virus core antigen

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Abstract

Several proteins of *Porphyromonas gingivalis* contain multiple copies of a 47 amino acid conserved repeated sequence. A fusion protein was constructed in which the *P. gingivalis* peptide was fused to the carboxy terminus of the hepatitis B core protein. This fusion protein was expressed in *Escherichia coli*, purified, and used to vaccinate mice that were later challenged with *P. gingivalis* W83 using the mouse abscess model. Although the mice were not protected against bacterial challenge, Western blot analysis showed that sera from the mice and from rabbits immunized with the fusion protein reacted with a number of vesicle proteins from *P. gingivalis* W83. These data suggested that this peptide is recognized by the host’s immune system but that the antibodies are not protective. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

*Porphyromonas gingivalis* has been implicated as a causative agent of periodontal disease, particularly in the etiology of adult-onset periodontitis. Multiple virulence factors have been attributed to this organism, and the most well studied of these are the gingipains, a family of cysteine proteases with specificity for arginine-X or lysine-X peptide bonds [1]. Allelic exchange mutants defective in either the arginine or lysine gingipain have reduced virulence in a mouse abscess model [2,3]. A 47 amino acid conserved repeated sequence (CRS), corresponding to amino acids 865–911 from Rgp-1 (A55426), is found in both cysteine proteases as well as in several other proteins (Fig. 1).

The prevalence of the CRS in proteins related to virulence led us to investigate the possibility that immunization with this 47 amino acid peptide might be protective against subsequent bacterial challenge in the mouse abscess model [4]. We chose to make a fusion protein with hepatitis B virus core protein. This protein, with additional peptides of various lengths fused to its carboxyl terminus, can be produced in *Escherichia coli* and self-assembles into the
hepatitis B virus (HBV)-like nucleocapsid particle. This particle formation increases the fusion protein’s immunogenicity and simplifies its purification [5]. In this study, we have used the CRS, fused to the core protein of HBV, as a vaccine in mice.

2. Materials and methods

2.1. Bacterial strains and plasmids

*P. gingivalis* W83 was cultivated as previously described [3]. The cloning vector, pHBVMM, and the purified recombinant hepatitis B virus core antigen (HBVcAg) were gifts from Darrell Peterson (Department of Biochemistry and Molecular Biophysics, Virginia Commonwealth University).

![Fig. 1. Alignment of conserved repeated amino acid sequences from various proteins of *P. gingivalis*. Uppercase letter in the consensus sequence indicates 100% conservation in all genes. The *P. gingivalis* strain designation is provided if a gene has been sequenced from more than one strain, and the number indicates which copy of the CRS within the protein is shown, with numbering proceeding from amino to carboxy end. The putative fibronectin-binding motifs introduced in Table 1 are shown here in boxes. Rgp1, RgpA, PrpRI, and PrpR are arginine-specific proteases. Kgp, PrtK, and PrtP are proteases with lysine specificity. HagA and HagD are hemagglutinins and Tla is a TonB-linked adhesin. The primary references for the listed sequences can be found through GenBank, using the accession numbers provided. Sequence analyses were performed using the Genetics Computer Group (Madison, WI, USA) and BLAST programs (http://www.ncbi.nlm.nih.gov/BLAST/).](http://www.ncbi.nlm.nih.gov/BLAST/).

2.2. Fusion protein construction

DNA encoding the 47 amino acid CRS was amplified by PCR from the chromosome of *P. gingivalis* W83. The primers, shown in the legend of Fig. 2, added the restriction sites BamHI and HindIII to the 5′ and 3′ ends of the sequence, respectively. A stop codon, TAA, was inserted downstream of the CRS coding region. Insert and vector were digested with BamHI and HindIII and both were purified from an agarose gel. The vector was further treated with shrimp alkaline phosphatase before being ligated with the 160 bp amplicon containing the conserved repeated sequence. The plasmid, with our amplified repeat sequence cloned at the 3′ end of the HBVcAg gene, was electroporated into *E. coli* TB1 cells and clones were selected by their resistance to
ampicillin. One of the clones, V2484, was chosen to produce the fusion protein (HBV-Pg protein).

2.3. Purification of the fusion protein

Twenty-four liters of strain V2484 were grown in a New Brunswick Fermentation system. The cells were harvested and HBV-Pg protein was purified to homogeneity using a previously published method [6]. For use in animals, HBV-Pg protein was further purified using an FPLC Mono Q column, equilibrated with 50 mM NaPO₄ buffer, pH 6.8. Presence of the fusion protein was determined using an ELISA which employed an antibody produced against HBVcAg (provided by D. Peterson) [6]. Protein concentration was determined using the DC Protein Assay kit (Bio Rad).

2.4. Polyclonal antibody production in rabbits

0.5 mg of the purified HBV-Pg protein in PBS was mixed with Freund’s Complete Adjuvant and used to immunize two female, white New Zealand rabbits. Rabbits were boosted after 6 weeks with 0.3 mg HBV-Pg protein mixed with Freund’s Incomplete Adjuvant. Two weeks following the boost, sera were collected from the rabbits, and the IgG fraction was purified with Affi-Gel Blue DEAE (Bio Rad) using the manufacturer’s protocol.

2.5. Immunization of mice and challenge with P. gingivalis W83

Female Balb/c mice (8–10 weeks) were divided into six groups of five mice each. Groups A and B received no immunization. Groups C and D were immunized with 40 µg HBV-Pg protein per mouse. HBV-Pg protein, in PBS, was mixed with alum (4 µg) and injected into each mouse on top of both shoulders. After 2 weeks, the mice were boosted with 30 µg protein mixed with 2 µg alum and injected as before. Groups E and F were immunized with 31 µg of HBVcAg mixed with 4 µg alum at the first immunization and 22 µg mixed with 2 µg alum at the boost. These amounts corresponded to the amount of HBVcAg that groups C and D received as part of the HBV-Pg protein. One week later, blood was collected from mice in groups B, D, and F, and sera were prepared and stored at −20°C.

The mouse abscess model was used to determine if immunization with HBV-Pg protein provided protection against infection with P. gingivalis W83 [4]. The bacteria were grown and prepared as described elsewhere [3]. Groups B, D, and F were challenged 21 days after immunization with subcutaneous injections of 1 × 10¹⁰ P. gingivalis W83 cells in 0.1 ml of sterile PBS at two sites on the dorsal surface. Groups A, C, and E were challenged 40 days post-immunization.

2.6. Evaluation of antibody production in rabbits and mice

Polyclonal antibodies from rabbit and mouse were screened for reactivity against the HBV-Pg protein and vesicle protein preparations from P. gingivalis W83, prepared as previously described [3]. Purified HBV-Pg protein or vesicle proteins containing 1 mM Nα-p-tosyl-l-lysine chloromethyl ketone (TLCK) were separated on 12% polyacrylamide gels at 200 V, and the proteins were transferred to nitrocellulose membranes using procedures described elsewhere [7]. The membranes were blocked in 2% (w/v) non-fat dry milk before being exposed to the primary antibody for 16 h (diluted 1:600 for rabbit and 1:500 for mouse). Following extensive washes in TBS containing 0.05% Tween, the blots were incubated for 1 h with the appropriate alkaline phosphatase-conjugated secondary antibody (diluted 1:2000). After washing, the blots were developed using Western Blue Stabilized Substrate (Promega). All buffers and wash solutions contained 1% (w/v) sodium azide.

3. Results and discussion

3.1. Production of HBV-Pg fusion protein

The HBV-Pg protein was constructed as shown in Fig. 2, expressed in E. coli TB1, and purified to homogeneity. Generally, the amount of protein produced was at least 20 mg l⁻¹. A total of 400 mg of purified protein was obtained for further studies.
3.2. Immunization of mice and challenge with *P. gingivalis* W83

The mice in groups D and F, immunized with HBV-Pg protein and HBVcAg, respectively, produced antibody directed against these proteins as determined by ELISA. Levels of antibody against HBV-Pg protein were 43- and 42-fold higher in the mice of groups D and F, respectively, than in the non-immunized group B mice. Antibody that reacted with HBVcAg was present in even greater amounts. Groups D and F mice had levels 174-fold and 146-fold higher, respectively, than group B mice (data not shown). However, when the mice were challenged with *P. gingivalis* W83, no difference was observed between the control group with no immunization and the experimental groups. All challenged mice were dead within 48 h. Increasing the time period between immunization and challenge (40 versus 21 days) did not affect our experimental outcome.

Previous studies have shown that fusion proteins containing various peptides fused to the carboxyl end of HBVcAg elicited immune responses in rabbits which were the same as those elicited with the wild-type, implying that the peptide neither enhances nor hinders the immune response. These same studies have shown that high titers of antibodies specifically directed against the peptide fused to the HBVcAg were obtained [5]. In our experiments, the mice in group D lacked antibody that reacted more strongly to the HBV-Pg protein than to HBVcAg alone (data not shown), and it could be this lack of a strong immune response to the CRS portion of the HBV-Pg fusion protein that resulted in our immunized mice being as susceptible to *P. gingivalis* W83 challenge as the non-immunized or the HBVcAg-immunized groups.

Because the CRS occurs within multiple *P. gingivalis* proteins, we hypothesized that this peptide might be a vaccine candidate. However, we were unable to show any protection when mice were immunized with the CRS fused to the core antigen of HBV. Many of the proteins containing the CRS have more than one copy of the sequence. While multiple copies of the CRS were present as part of the HBV nucleocapsid particle, there were single copies of the CRS in each fusion protein. Perhaps the multiple copies of the CRS within the *P. gingivalis* proteins contribute to structural features that our fusion protein lacks. For example, there might be conformational epitopes formed by copies of the CRS at precise locations within one protein. The importance of multiple copies of this sequence is alluded to with the DFEEDT and EVEDT/S motifs which are found within the CRS. Both motifs appear in repeated sequences found within fibronectin-binding proteins in *Staphylococcus aureus*, *Streptococcus dysgalactiae*, and *Streptococcus equisimilis* [8] (Table 1). Similar sequences are found in the CRS of *P. gingivalis*, mostly as TFEEDG [9] and EVKYT, as shown in Fig. 1. Thus, both the number of copies and the location of multiple copies within a single protein

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**Porphyromonas gingivalis** W83 genomic DNA

PCR amplification of repeated sequence from hemagglutinin domain of *rgp-1*

160 bp CRS Amplicon + pHBVMM

BamHI HindIII

lacZ HBV CRS lacZ

pVA2484

Fig. 2. Fusion protein construction. PCR primers were prepared using the sequence of *rgp-1* (U15282). Forward primer, 5′-CGG GGA TCC ATG GT TAT ACC TAC ACG GTG-3′; reverse primer, 5′-AGT AAG CTT TTA ACA TTT CTT CGG AGA TAC GC-3′. Added restriction sites for BamHI and HindIII are italicized. The sequence encoding the CRS was fused to the 3′ end of the HBV core antigen gene. The final product was a β-galactosidase-HBVcAg-Pg CRS fusion protein as indicated by the arrow in the figure.
relative to one another might affect the function of antibodies raised against the CRS.

3.3. Reactivity of polyclonal antisera raised against the HBV-Pg fusion protein

Polyclonal antibodies were collected from the sera of immunized rabbits and mice. Western blot analysis showed that the IgG purified from the rabbits reacted with both the HBV-Pg protein and with several vesicle proteins from \textit{P. gingivalis} W83. A representative experiment using serum from one rabbit is shown in Fig. 3. In lane 2, the 75.9 kDa protein could be the 76 kDa protein found in the culture supernatant of an \textit{rgpA} mutant and the wild-type strain which was immunoreactive with anti-Arg-gingipain IgG [10]. The protein shown by the single band at 62.5 kDa could be the 60 kDa protein described as the Lys-gingipain protease while the 48 kDa protein might be the 50 kDa arginine-specific protease, gingipain [11]. Other prospects for the identity of the 48 kDa protein include the 48 kDa lysine-specific protease [12]. Any of these possibilities are plausible because both the arginine- and lysine-specific proteases contain multiple copies of the CRS which was recognized by our antisera. In lane 1, the purified HBV-Pg protein produced multiple immunoreactive bands. Those proteins which are larger than 26 kDa are probably various multimeric forms due to the HBVcAg portion of the fusion protein while those smaller are presumably degradative products.

Similar results were seen using polyclonal sera obtained from the immunized mice. A representative Western blot is shown from each group of mice. Antisera from groups B and F, unimmunized and immunized with HBVcAg, respectively, did not react with \textit{P. gingivalis} W83 vesicle proteins (Fig. 4, lanes...
B1, F1). Group B antiserum also showed no immunoreactivity with the HBV-Pg protein while the antiserum from the mouse immunized with HBVcAg (group F) did indeed react with the fusion protein (lane F2). Serum from group D, immunized with HBV-Pg protein, showed reactivity with several proteins migrating between 64 kDa and 87 kDa in the *P. gingivalis* vesicle proteins (lane D2). This is similar to results obtained when mice were immunized with a 25 amino acid synthetic peptide, of which the first 18 amino acids overlap the carboxy end of the CRS. While antibody against this 25 amino acid peptide was produced, immunization with the peptide did not protect against subsequent bacterial challenge [13]. Other attempts to immunize mice and provide protection against bacterial challenge with *P. gingivalis* have been more successful. Chen et al. immunized mice with whole cell suspensions and found that immunization with either live or pasteurized cells of an invasive strain of *P. gingivalis* protected against subsequent challenge with the same strain [14]. Other experiments have shown that production of cysteine protease activity by *P. gingivalis* is related to the size and type of lesions produced and to the overall virulence of the organism in the mouse abscess model (reviewed in [15]). Deslauriers et al. used the mouse chamber model to demonstrate that immunization with a subsequence peptide of the fimbriillin molecule could provide protection against bacterial challenge 40 days post-immunization [16].

It is interesting to note that the presence of antibody directed against the CRS was not protective in the mice. We used HBV-Pg protein and ELISA to screen periodontal patient sera (kindly provided by VCU Clinical Research Center for Periodontal Diseases) to determine if antibody against the CRS was present. Of the 45 sera tested, 39 had varying levels of IgG directed against the HBV-Pg protein (data not shown). However, there was no correlation between the level of antibody against HBV-Pg protein and attachment loss (data not shown).

We have shown that polyclonal rabbit and mouse antisera generated against the HBV-Pg protein cross-react with several *P. gingivalis* W83 vesicle proteins, indicating the presence and cross-reactivity of the CRS in these proteins. The sizes of some of these proteins correspond to virulence-associated proteins which have already been described [10–12]. Others which appear to contain the CRS have not yet been characterized. The prevalence of this CRS had suggested that it may serve as a target for vaccine development as this would affect several key proteins of the organism that have been shown to be involved in virulence [10,17–19]. These data further suggest the importance of the CRS to recognition of *P. gingivalis* by the host immune system since animals immunized with the HBV-Pg protein elicited antibodies that reacted with multiple *P. gingivalis* vesicular proteins and periodontal patient sera contained antibodies which reacted with HBV-Pg protein. Other chimeric vaccines, perhaps with multiple copies of the CRS, may prove more successful, or the use of this or other chimeras may prove successful in other animal models.

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