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Sera from mice immunized with DNA encoding *Porphyromonas gingivalis* catalytic or adhesin part of HRgpA inhibit degradation of human fibronectin by HRgpA

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Gingipains are potent virulence factors of *Porphyromonas gingivalis* and are likely to be associated with the development of periodontitis. It is, therefore, suggested that gingipain inhibition by vaccination could be a useful therapy for adult periodontitis. This study investigated the ability of antibodies raised against the catalytic part and the adhesin/haemagglutinin part of HRgpA to prevent haemagglutination and fibronectin degradation caused by *P. gingivalis*. We constructed two DNA vaccines, one containing the adhesin part of HRgpA and one with the catalytic part of HRgpA. BALB/c mice were immunized intramuscularly with either catalytic-part-encoding plasmids, adhesin-part-encoding plasmids or empty control plasmids. Sera from mice immunized with the catalytic vaccine or the adhesin vaccine each showed inhibition of human fibronectin degradation. A DNA vaccine encoding the adhesin or catalytic part of HRgpA induces responses that inhibit the degradation of molecules important for the structure and function of gingival and bone tissues.

Key words: DNA vaccine; fibronectin; HRgpA; *Porphyromonas gingivalis*

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Periodontitis is a chronic inflammatory condition that can result in tooth loss and bone and gingival tissue destruction. The disease affects between 30% and 40% of the dentated population with about 13% being seriously affected (8, 15).

Porphyromonas gingivalis, a gram-negative, anaerobic, asaccharolytic bacterium, has been implicated as a major causative pathogen in the initiation and progression of chronic periodontitis (7, 29). This bacterium has an obligate need for iron, which it acquires preferably in the form of haemine from haemolysed erythrocytes because of its lack of a siderophore system (2, 10).

P. gingivalis produces a variety of virulence factors including some proteolytic enzymes, the gingipains, which have been shown to be important for establishment of the bacterium in the oral environment. These gingipain-related factors include provision of nutrients for the bacteria, host tissue destruction and host immune response modulation (18). Two main types of gingipains are found which are encoded by three genes: *rgpA*, *rgpB* and *kgp* (4). The *rgpA* and *rgpB* genes encode the arginine-specific proteases and *kgp* encodes the lysine-specific protease. The product of *rgpA*, HRgpA, and the product of *kgp*, Kgp, are polyproteins with C-terminal adhesins. These polyproteins are processed by cell Rgp and Kgp to several sub-domains (1, 27). The domains with proteolytic activity will form complexes with the adhesin domains through binding motifs called adhesin-binding motifs (ABMs) (1, 27). The product of *rgpB*, RgpB, lacks this ABM and will consequently not bind to adhesins from the RgpA/Kgp autoproteolytic processing.

While the adhesin parts of HRgpA and Kgp are highly conserved (1) the catalytic parts share only limited identity (27%) scattered through the peptide, except for 30 amino acids at the C-terminal which are identical (9). The proteolytic parts of HRgpA and RgpB have a large degree of similarity but RgpB lacks the ABM, as mentioned above.

The haemagglutination activity of the bacterium has been located to ABMs in the adhesin part of the gingipains (25).The adhesin part also has binding activity to several host proteins like fibrinogen, fibronectin, laminin, haemoglobin and collagen type V (18), which makes it important with regard to increasing the degradation of these host proteins (24). In addition, the adhesin part has been shown to increase the binding of *P. gingivalis* to epithelial cells (3). This increases its ability to colonize the gingival crevice.

It has been known for more than a decade that injection of protein-encoding expression plasmids in animals results in expression of the encoded protein (32). This knowledge has been used to create a new immunization strategy, nucleic acid immunization (30). By immunizing with protein-encoding DNA or RNA the expressed protein will induce an immune reaction in the host. Most studies have concentrated on DNA immunization. DNA vaccines are relatively simple to design and inexpensive to manufacture. They have a built-in adjuvant effect because unmethylated DNA is immunostimulatory for mammals. Also, regions like the CpG islands in the ampicillin-resistance gene are immunostimulatory (22).

It has previously been shown that the adhesin part of HRgpA can be expressed in transfected eukaryotic cells and that the eukaryotic expressed adhesin behaves in the same way as the one expressed in *P. gingivalis* (23, 31). When we immunized rats with expression plasmids encoding the adhesin part, the rats expressed the adhesin in skeletal muscles and developed antibodies specific for the adhesin (31). In the present study we performed DNA immunization with constructs encoding the adhesin part or the catalytic part of HRgpA and measured the inhibitory effect of sera from immunized mice on the proteolytic and haemagglutinating activity of *P. gingivalis.*

Material and methods

Bacterial strain and culture conditions

P. gingivalis W50 (ATCC 53978) was transferred to anaerobic modified chopped meat medium (ATCC culture medium 1490) and incubated to log phase of growth under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) at 37°C.

Construction of plasmid DNA for immunization

Genomic DNA from *P. gingivalis* W50 was isolated from cultivated bacteria as previously described (31). Primers were constructed to amplify an 864-base-pair (bp) fragment of the β-adhesin part and a 1200-bp fragment of the catalytic part of the *rgpA* gene. The primers used were as follows; for the adhesin fragment the sense primer was 5′-ATAATGGAAGGTGGTG-GAAGCGATTAC-3′ and the antisense primer was 5′-CTCGAACGTTTC-CGTGAAGTCTGCGCG-3′; for the catalytic fragment the sense primer was 5′-CACCATGTACACACCGGTAGAGG-AAAAA-3′ and the antisense primer was 5′-GGCATCGCTGACTGACAGAAG-3′. The fragments were amplified using *Pfu* polymerase (Promega, Madison, WI) with the following parameters: denaturation at 94°C for 1.5 min, followed by four cycles of denaturation at 94°C for 1 min; annealing at 57°C for 30 s, and extension at 68°C for 2 min, followed by 37 cycles of denaturation at 94°C for 1 min; annealing at 62°C for 30 s, and extension at 68°C for 2 min. This was followed by final extension at 68°C for 10 min. The amplified fragments were inserted into the pcDNA3.1 directional TOPO expression vector (Invitrogen, Carlsbad, CA). The sequence of the cloned DNA was checked by sequencing (Big Dye Cycle Sequencing kit, Applied Biosystems).

Mouse immunization

The DNA immunization was performed in male BALB/c mice fed on standard mouse chow and kept in groups of 10 animals. At the time of the first immunization the animals were 6 weeks old. Three groups of 10 animals were used. One group was immunized with plasmid containing the adhesion fragment, one group was immunized with plasmid containing the catalytic fragment and one group was immunized with pcDNA3.1 without any insert. Each animal was immunized intramuscularly in the femoral muscle with 100 μg DNA dissolved in 100 μl phosphate-buffered saline (PBS). The mice were immunized at weeks 0, 3, 6 and 7. Blood samples were obtained from mice at weeks 0 (before the first immunization), 2 (before the second immunization), 5 (before the third immunization) and 8 (when mice were sacrificed). The experiments were performed with the approval of the Norwegian State Board for Biological Experiments with Living Animals.

Detection of *P. gingivalis* HRgpA mRNA in immunized animals

Femoral skeletal muscle was dissected from the immunized animals. The muscle tissue was homogenized and total RNA was extracted and isolated using TRIzol (Gibco, Invitrogen, Carlsbad, CA) following the procedure described by the manufacturer. To avoid contamination of the samples with plasmid DNA from the immunizations, purified RNA was treated with a Turbo DNase Kit (Ambion Austin, TX). Total RNA (1 μg) was amplified by reverse transcription–polymerase chain reaction (RT-PCR) using an Access RT-PCR kit (Promega, Madison, WI). The primers used for the RT-PCR were as follows: for the adhesion RNA: 5′-GTATGCGCACAGG-ATGCTAA-3′ (sense) and 5′-TATTTCGT-ACCTGCGGGAAG-3′ (antisense), and for the catalytic RNA: 5′-CAGCTTACCAA-CAGCAACCA-3′ (sense) and 5′-GGGT-GTTTTTCGCACAGAAT-3′ (antisense). Reverse transcription was performed at 48°C for 45 min. Amplification was performed with 40 cycles of 94°C for 1 min, 57°C for 30 s and 68°C for 30 s. This was followed by a final elongation period of 72°C for 10 min. The amplification product was displayed on an ethidium bromide-stained 2% agarose gel.

Enzyme-linked immunosorbent assay (ELISA)

P. gingivalis bacteria were grown as described above, washed three times in PBS and sonicated three times for 30 s on ice. A 96-well Maxi Sorp plate (Nunc, Roskilde, Denmark) was coated with sonicated bacteria, 40 μg bacterial sonicate in 100 μl 50 mM carbonate buffer (pH 9.6) per well, and incubated at 4°C overnight. The wells were blocked with 3% skimmed milk in PBS and incubated at room temperature for 1 h, 100 μl of twofold serial dilutions of mouse serum in PBS was incubated at room temperature for 1 h, and 100 μl peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) antibody (Sigma, St Louis, MO) was then added and incubated at room temperature for 1 h. The wells were washed three times with PBS-Tween (0.05% Tween) after each step. Substrate (TMB from Sigma, T0440) was added and absorbance was measured at 665 nm (A_{665}). The serum

dilutions resulting in an A_{655} twice as high as that of preimmunized sera diluted 1 : 5 was used as an end-point titre.

Isotyping of specific antibodies

Microtitre plates (96-well Maxi Sorp) were coated with sonicated *P. gingivalis* bacteria as described above. The plates were then incubated with sera from immunized mice diluted twofold from 1 : 10 in PBS-Tween and incubated for 1 h at room temperature. Thereafter the wells were incubated with goat anti-mouse IgG1, goat anti-mouse IgG2a (all goat anti-mouse IgGs were from the Sigma mouse antibody isotyping kit, ISO2). The ISO2 antibodies were diluted 1 : 1000 in PBS-Tween and incubated for 1 h at room temperature. All wells were then incubated with peroxidase-conjugated rabbit anti-goat IgG (Sigma A5420). After each step the wells were washed three times with PBS-Tween. The wells were incubated with 100 µl substrate (TMB from Sigma, T0440) at room temperature for 30 min and the reaction was then stopped with 100 µl 0.5 M H_2SO_4. The absorbance was measured at 450 nm (A_{450}) and the end-point titres were defined as the dilution resulting in an absorbance value twice as high as that of preimmunized sera with a cut-off of $A_{450} = 0.2$.

Preparation of proteases

A crude preparation of proteases was made as described by Ono et al. (19). Briefly, supernatant from a 72-h culture of *P. gingivalis* ATCC 53978 was collected by centrifugation at 8000 ***g*** at 4°C for 15 min. Proteins were precipitated by slow addition of ammonium sulphate to 80% saturation with gentle stirring at 4°C overnight. The precipitate was collected by centrifugation at 25 000 ***g*** at 4°C for 30 min, dissolved in a small volume of PBS, pH 7.3, and dialysed (molecular weight cut-off 12 000–14 000) twice for 3 h each time against deionized water and once for 6 h against PBS, pH 7.3 at 4°C overnight.

Protease degradation of human fibronectin

The *P. gingivalis* protease (0.6 µg) was incubated with serum from immunized mice, twofold diluted in PBS containing 1 mM dithiothreitol, for 1 h at room temperature. Serum dilution ranged from 1 : 6 to 1 : 1536. Protease incubated with PBS/dithiothreitol without serum was used as control. Human fibronectin (10 µg) was then added to each tube and the mixture was incubated for a further 1 h at 37°C. The total incubation volume was 20 µl. The cutting products were kept at −20°C until use in immunoblot analysis. Fibronectin incubated at 4°C overnight with anti-fibronectin was used as the positive control.

Immunoblot analysis

Protease/fibronectin samples incubated with serum dilutions ranging from 1 : 48 to 1 : 768 were used in the immunoblot analysis. Sample buffer containing mercaptoethanol was added for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The samples were heated at 95°C for 5 min and loaded on a SDS–PAGE gel (4% and 12% acrylamide in stacking and separation gels, respectively). After separation the proteins were transferred to a nitrocellulose membrane by electrophoresis at 4°C, 100 V for 1 h. After this the membrane was washed twice with Tris-buffered saline (TBS) and blocked for 1 h with 3% dried skimmed milk diluted in TBS. The membrane was then washed twice for 5 min each time with TBS-Tween (0.05%) and incubated for 1 h with polyclonal rabbit anti-fibronectin (Sigma) diluted 1 : 2000 in TBS-Tween followed by one 5-min and two 10-min washes with TBS-Tween. The membrane was incubated with alkaline phosphatase-conjugated goat anti-rabbit (Sigma), diluted 1 : 2000 in TBS-Tween, washed once for 5 min and twice for 10 min in TBS-Tween and incubated in substrate (Sigma) for detection. To confirm the identity of the fibronectin on the blot, anti-fibronectin serum was preadsorbed with fibronectin. Anti-fibronectin diluted 1 : 2000 in TBS-Tween was incubated overnight with 20 µg fibronectin at 4°C. The preadsorbed serum was then used as a primary antibody in immunoblotting. After detection the membranes were scanned and analysed on GelDoc software (Bio-Rad, Hercules, CA).

Haemagglutination

Proteins were purified from *P. gingivalis* culture media as described above. The amount of purified protein and sheep erythrocyte used in the assay was determined by preliminary testing. A 25-µl sample of purified protein (200 µg/ml) was incubated with 12.5 µl serum from the immunized mice for 30 min at room temperature in a round-bottomed microtitre plate. Mouse sera were diluted twofold with PBS before being mixed with the purified *P. gingivalis* protein. Then, 25 µl 1% sheep erythrocyte suspension was added and the wells were incubated for 3 h at room temperature. The wells were then inspected for haemagglutinating activity.

Statistics

The data were tested with Student's *t*-test and *P*-values of $\leqslant 0.05$ were considered statistically significant. Data are presented as means ± SE.

Results

Expression of the HRgpA in immunized animals

The immunized animals were investigated for HRgpA in femoral skeletal muscle cells by RT-PCR. All mice immunized with the plasmid encoding the catalytic part of HRgpA were positive for mRNA encoding this part of the protein. All mice immunized with the plasmid encoding the adhesin part of HRgpA were positive for mRNA encoding this protein. Mice immunized with the pcDNA3.1 control plasmid were negative for both catalytic- and adhesin-encoding mRNA (data not shown).

Detection of HRgpA-specific antibodies in immunized mice

The increase in HRgpA-specific antibodies in the sera from immunized animals was measured by ELISA and is presented as end-point titres (Fig. 1). There was a significant increase in end-point titre in sera from mice immunized with either the adhesin part or the catalytic part compared

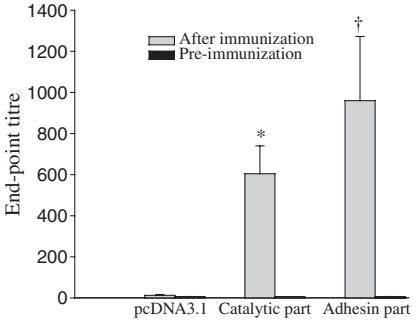

Fig. 1. Increase of HRgpA-specific antibodies after immunization as ELISA end-point titres. The results are presented as means ± SE. *Catalytic part vs. pcDNA3.1 $P < 0.001$, †adhesin part vs. pcDNA3.1 $P < 0.004$.

to the preimmunization sera (960 ± 312 compared to 6.25 ± 1.25, $P < 0.004$ for adhesin-immunized animals and 604 ± 135 compared to 5 ± 0, $P < 0.001$ for catalytic-part-immunized animals). There was no significant difference in the titre of adhesin-part-immunized and catalytic-part-immunized animals ($P > 0.2$). There was no significant increase ($P > 0.2$) in the sera from pcDNA3.1-immunized animals compared to preimmunization sera.

Detection of different IgG subclasses

We analysed the IgG1 and IgG2a subclasses in sera from the immunized mice (Fig. 2). We found the titre for both IgG1 and IgG2a to be increased in both adhesin-part- and catalytic-part-immunized animals compared to the preimmunization sera. The antibody response was, however, dominated by IgG2a both in adhesin- and catalytic-part-immunized animals (adhesin-immunized animals, IgG1: 148 ± 32, IgG2a: 593 ± 129; catalytic-part-immunized animals, IgG1: 89 ± 15, IgG2a: 462 ± 56). In pcDNA3.1-immunized animals we found no significant difference between the titre of IgG1 and IgG2a (12 ± 2 and 9 ± 2 for IgG1 and IgG2a, respectively, $P > 0.04$). We therefore identified the antibody immune response to both the adhesin and catalytic parts to be Th1 dominated ($P < 0.006$ and $P < 0.001$ for adhesin and catalytic parts, respectively).

Degradation of fibronectin by *P. gingivalis* proteases

The activity of the *P. gingivalis* proteases was confirmed by subjecting human fibronectin to degradation of the purified proteins. This was visualized by immunoblotting. The results from the immunoblotting showed that the *P. gingivalis* proteins degraded the fibronectin. The specificity of the antibody was shown by inhibition of antibody recognition after adsorbance of the antibody with fibronectin (data not shown).

Inhibition of fibronectin degradation by sera from immunized animals

The ability of the sera from the different groups of immunized animals to inhibit protease degradation of human fibronectin was studied and visualized by immunoblotting. The results are presented as the serum dilution at which the sera protected fibronectin against proteolytic degradation (Fig. 3). There was a significant increase in end-point titre for both sera from catalytic-part-immunized and adhesin-part-immunized animals compared to pcDNA3.1-immunized animals ($P < 0.02$ and $P < 0.002$ for catalytic-part- and adhesin-part-immunized animals, respectively). Sera inhibited fibronectin degradation up to serum dilutions of 1 : 75 (±8) for pcDNA3.1-immunized mice, 1 : 356 (±107) for catalytic-part-immunized mice and 1 : 400 (±93) for adhesin-part-immunized animals.

Inhibition of haemagglutination by sera from immunized animals

There was no significant difference in haemagglutination inhibition between sera from the three immunization groups (Fig. 4). End-point titres were 142 ± 23 for pcDNA3.1, 149 ± 21 for the catalytic-part-immunized and 149 ± 21 for the adhesin-part-immunized animals ($P > 0.8$ for catalytic vs. pcDNA3.1-immunized animals, and $P > 0.8$ for adhesin vs. pcDNA3.1-immunized animals).

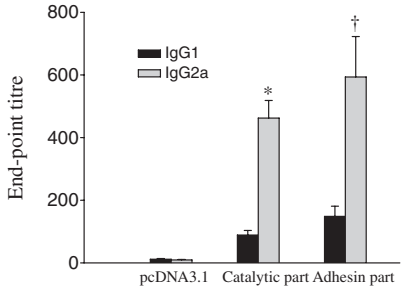

Fig. 2. Increase in HRgpA-specific IgG1 and IgG2 after immunization as ELISA end-point titres. The results are presented as means ± SE. *Catalytic part IgG2a vs. IgG1 $P < 0.001$, †adhesin part IgG2a vs. IgG1 $P < 0.006$. The immune response is therefore Th1 dominated.

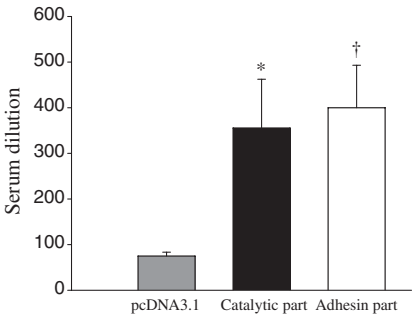

Fig. 3. Serum inhibition of fibronectin degradation. The results are means ± SE of the last serum dilution inhibiting fibronectin degradation. *Catalytic part vs. pcDNA3.1 $P < 0.02$, † adhesin part vs. pcDNA3.1 $P < 0.002$.

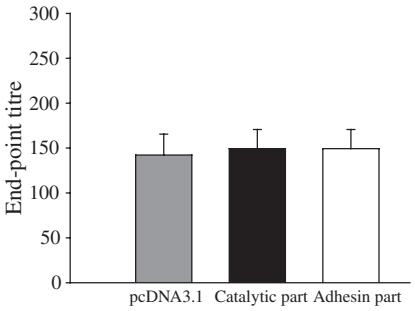

Fig. 4. Serum inhibition of haemagglutination. The results are means ± SE of the last serum dilution inhibiting haemagglutination. There was no significant difference in the ability to inhibit haemagglutination between the different immunization groups.

Discussion

In the present study we demonstrate that intramuscular DNA immunization against the adhesin part or catalytic part of HRgpA induces a specific immune response that inhibits the proteolytic degradation of fibronectin (Figs 1 and 3). This inhibition is achieved both with adhesin part immunization and catalytic part immunization. There was, however, no increased inhibition of the haemagglutinating activity by the sera from the immunized animals (Fig. 4). This contradicts what was previously observed by Yonezawa et al. (33). The reason for the diverging results could be the different immunization strategies used and/or differences in plasmid constructs. Yonezawa et al. used a gene gun for the immunization. This is a method that usually induces a T helper type 2 (Th2) response. We used intramuscular immunization and previously have shown this to induce a Th1 response in rats (31). This finding was confirmed in the present study where we found a higher increase in the IgG2a level compared to the increase in the IgG1 level (Fig. 2), indicating that the immune response is Th1 dominated. The differences in plasmid constructs may affect the three-dimensional folding of the expressed protein antigen and thereby the binding of B cells to the antigen. This is contrary to what we have previously found, when the three different adhesin-encoding plasmids were used for expression in COS-7 cells (31). The cells were screened for adhesin expression by an antibody known to inhibit haemagglutination (11). The epitope recognized by this antibody contains the sequence PVQNLT, which had previously been identified as the haemagglutinating domain of the adhesin part of the protein (25). All three

different versions of the adhesin were expressed and recognized by the monoclonal antibody 61BG1.3. The plasmid used in the present study was the one found to be most efficiently expressed in the COS-7 cells (31). Even though the haemagglutination-inhibiting antibody did recognize the protein in cell culture, DNA immunization with the same expression plasmid did not induce a haemagglutination-inhibiting immune response. In addition to the PVQNLT haemagglutinating motif, another motif with haemagglutinating activity has been identified by Kelly et al. (11). This motif is not recognized by the haemagglutination-inhibiting antibody and is localized between the two different PVQNLT-containing regions in the adhesin. This region was not investigated for haemagglutination activity by Shibata et al. (25). Kelly et al. also tested the PVQNLT-containing region for haemagglutinating activity but with negative results. However, the sequence of the peptide fragment used was not identical to that used by Shibata et al. and this might have influenced the haemagglutinating ability of the fragment. It is possible that the haemagglutinating domain of the adhesin consists of both areas and that they are brought together in the folded protein. Yonezawa et al. (33) used the whole HRgpA-coding sequence for immunization while we have used the two parts separately. We also used only a part of the adhesin in our immunization. If the protein is misfolded this can affect the immunogenicity of the different parts of the protein and the ability of the protein to induce a haemagglutination-inhibiting immune response. It has previously been shown that the adhesin part is important for the degradation of several substrates like fibronectin, fibrinogen and laminin (20). The adhesin part binds to these substrates and promotes the proteolytic degradation by the catalytic part. HRgpA is found to be 12 times more effective in degrading fibronectin and its integrin-α_5 β_1 receptor than the catalytic part alone (24). HRgpA was found to be more active in fibronectin degradation than Kgp. We observed that sera from both adhesin-part-immunized and catalytic-part-immunized animals inhibited fibronectin degradation (Fig. 3). There was no significant difference in the ability to inhibit the degradation. We used a crude protease preparation for the fibronectin degradation assay. The preparation therefore contained several types of proteases, including all types of gingipains. Sera from the catalytic-part-immunized animals could therefore only partially inhibit the degradation. The adhesin sequence -FEED- is connected to the fibronectin binding (17, 21). The -FEED- sequence has also been identified as a fibronectin-binding motif in *Staphylococcus aureus* (13, 26). In *P. gingivalis* the fibronectin-binding motif is found both in the adhesin part of HRgpA and Kgp. It is also found in the haemagglutinin Hag A. Hag A is thought to be part of the HRgpA–Kgp complex (17) and is therefore important for fibronectin degradation. Immunization with HRgpA-encoded adhesin therefore has the ability to induce reduction of both HRgpA and Kgp degradation of fibronectin. This is probably the reason why sera from adhesin-part-immunized animals protected as well as sera from the mice immunized with the catalytic part. Antibodies directed towards the -FEED- binding region have also been shown to block the binding of the HRgpA–Kgp complex to fibrinogen and collagen type V (17). The same region seems to be important for binding to laminin (20). Even though both HRgpA and Kgp contain the -FEED- sequence they have different affinities for the different substrates. The binding of the different substrates is therefore also probably dependent on the three-dimensional structure of the protein and not the primary structure alone.

Targeting of the HRgpA–Kgp complex to different host proteins is probably important for the ability of *P. gingivalis* to invade different host tissues. A vaccine that induces blocking of the ability to adhere to host cell protein receptors is therefore not only important for reducing the ability of *P. gingivalis* to induce periodontitis, but also for its induction of systemic diseases associated with periodontitis.

In periodontitis patients, antibodies towards the HRgpA–Kgp complex are predominantly directed towards the adhesin part of the molecule. The catalytic part seems to be poorly immunogenic (5, 14, 28). In a vaccination trial when mice were immunized with heat-killed *P. gingivalis* HRgpA and RgpB protein, only antibodies towards the gingipain adhesin part were found to protect against maxillary bone loss in an oral challenge model (6). In another study it was found that antibodies from mice immunized with the HRgpA–Kgp protein complex were directed towards the adhesin part and not towards the catalytic part (16). Immunization with peptides consisting of the histidine-containing site of the RgpA and Kgp catalytic sites induces a protective antibody response (17). We observed an antibody response in the mice immunized with the catalytic-protein-encoding expression plasmid. This antibody response reduced proteolytic degradation of fibronectin. DNA immunization therefore seems to be able to elicit an inhibitory response towards the catalytic part of HRgpA in contradiction to what is observed with HRgpA whole protein immunization. This is confirmed in other DNA immunization studies (12, 33). In a DNA vaccine, the antigenic protein is expressed by the host cells. This seems to induce a protein folding that is different from the one observed in *P. gingivalis* cells. In the DNA vaccine experiment the active site of the catalytic part seems to be more exposed and thereby induces a protective antibody response.

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