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Original

Inhibition of *Porphyromonas gingivalis* hemagglutinating activity by IgY against a truncated HagA

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Abstract: ***Porphyromonas gingivalis*** **has been implicated as an important pathogen in the development of periodontitis. Hemagglutinins have been identified as important adhesion molecules, allowing** ***Porphyromonas gingivalis*** **to adhere to gingival tissue cells, and to attach and lyse erythrocytes in order to uptake Fe ions as essential nutrition. One hemagglutinin, hemagglutinin A (HagA), has been molecularly cloned via functional screening for hemagglutinating activity. We previously cloned the gene encoding the 200-kDa cell-surface antigenic protein that was reacted by sera from periodontitis patients and was identified as a truncated protein of HagA by nucleotide sequence analysis. We further subcloned the gene encoding an 122-kDa protein (122k-HagA) which is a fusion protein composed of an 80-kDa truncated HagA containing the functional motif PVQNLT and a 42-kDa maltose binding protein. Passive immunization against infectious pathogens by specific antibodies produced from hen egg yolk antibody (IgY) has been extensively developed. In the present study, to develop passive immunotherapy against periodontal disease, we purified the recombinant 122k-HagA and used this to immunize hens and produce IgY. The purified IgY reacted with the recombinant 122k-HagA and the synthetic peptide containing PVQNLT, and inhibited hemagglutinating activity of** ***Porphyromonas gingivalis*****. Thus, the novel IgY may be useful in the development of a passive immunization against periodontal diseases caused by** ***P. gingivalis*** **infection. (J. Oral Sci. 48, 227-232, 2006)**

Keywords: IgY; *Porphyromonas gingivalis*; HagA; hemagglutinin.

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Introduction

Porphyromonas gingivalis has been implicated as an important pathogen in the development of periodontitis (1,2). The adherence of bacterial pathogens to host tissue cells is a crucial step in pathogenesis. Moreover, protoheme is an absolute requirement for the growth of *P. gingivalis* (3), and *P. gingivalis* possesses both hemagglutinating and hemolytic activities, as well as having several enzymes that promote colonization and aid the acquisition of hemin or iron from hemoglobin present in erythrocytes (4). Thus, hemagglutinins may be important for this microorganism not only for adherence to the gingival tissue cells but also to attach and lyse erythrocytes in order to survive.

Hemagglutinin A (HagA) has been molecularly cloned via functional screening for hemagglutinating activity from *P. gingivalis* (5). Within HagA's open reading frame four direct repeats were identified, and the repeat unit was thought to contain the hemagglutinin functional domain (6). HagA is known to be a potentially useful immunogen and elicits an immune response that may be protective against colonization of *P. gingivalis* (7).

In our studies of the pathogenesis of *P. gingivalis* and ways to protect against periodontal disease, we prepared a monoclonal antibody, MAb-Pg-vc, that was capable of

neutralizing the hemagglutinating activity of *P. gingivalis* (8). Moreover, we molecularly cloned a 130-kDa hemagglutinating domain (130k-HMGD) using MAb-Pg-vc and identified PVQNLT as the essential motif of the functional domain (9). Interestingly, there is extensive homology between 130k-HMGD and other abundant *P. gingivalis* proteins, including the proteases PrtP, PrtH, RGP-1, PrtR, RGP, KGP, and Arg1, and HagA and HagD in the genome database, now known as the *hag* gene family (9).

We previously constructed a gene library of *P. gingivalis* 381 and probed with sera from patients with severe periodontitis; this enabled us to successfully clone a 200-kDa antigenic protein (200k-AP) (10). The short peptide region of 200k-AP (11) and DNA sequences of 200k-AP (12) were identical to those in the N-terminal region of HagA and the *hagA* gene, respectively. Thus, our cloned gene encoding 200k-AP was identified as the *hagA* gene. It is noteworthy that HagA also possesses a PVQNLT stretch in repeat units (Fig. 1).

Since the gene clone harboring the 200k-AP gene exhibited poor recombinant protein production, in order to produce large amounts of proteins for immunotherapy, we tried to construct a subclone that yielded large quantities of recombinant protein. Recently, active immunization has been successfully established using *Escherichia coli*-expressed chimera immunogen composed of a 42-kDa maltose binding protein (MBP) with target antigen; the resulting immunogen is soluble (13) and gives a higher protection rate against infection (14,15). Therefore, we sucloned the 200k-AP gene into pMD160 which encoded the 122-kDa protein, designated 122k-HagA, which was a fusion protein composed of an 80-kDa truncated protein of HagA containing the functional short motif of PVQNLT

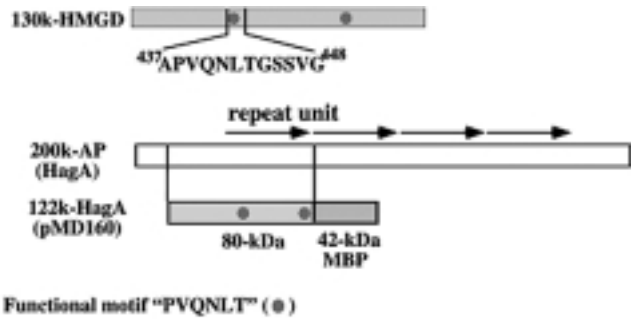

Fig. 1 Molecular map of 130k-HMGD, 200k-AP, and 122k-HagA. The functional short motif "PVQNLT" expressing hemagglutination activity was shown in 130k-HMGD, repeat unit of 200k-AP, and 122k-HagA. "APVQNLTGSSVG" was a synthetic peptide (peptide-437AP) spanning the 437 to 448 amino acid stretch of 130k-HMGD.

and a MBP (16). Molecular maps of 130k-HMGD, 122k-HagA, and 122k-HagA are shown in Fig. 1.

When developing an immunotherapy strategy, the biosafety of the antibody utilized is essential, and passive immunization approaches against oral infectious diseases have been developed with such safety in mind (17). Passive immunization therapies generally require the preparation of a large amount of antibodies when the oral administration route is utilized. Due to such demand, specific antibodies from hen egg yolk preparations (IgY) have recently attracted considerable attention (18).

In the present study, to develop passive immunotherapy against periodontal disease, we purified 122k-HagA and used it to immunize hens and produce specific IgY against 122k-HagA that was capable of neutralizing the hemagglutinating activity of *P. gingivalis.*

Materials and Methods

Cell culture and vesicle preparation

P. gingivalis 381 was grown in a brain-heart infusion - 0.25% yeast extract supplemented with hemin (5 μg/ml) and vitamin K (0.5 μg/ml). These cultures were incubated at 37°C in an anaerobic chamber containing 80% N_2, 10% H_2, and 10% CO_2, and the vesicle fraction was prepared as previously reported in the literature (19).

Production of IgY

Recombinant (r) 122k-HagA was prepared by a method reported previously (16). Rhode Island Red hens (200 days old) were intramuscularly immunized in both legs with purified protein (0.25 mg/ml/animal) using Freund's complete adjuvant (Difco Lab). Following the initial immunization, 2 booster injections were given at an interval of 2 weeks using Freund's incomplete adjuvant. One milliliter of blood was collected from each animal from the vein below the wing every 2 weeks following the first injection, and the serum was separated and stored at -30°C. All eggs laid were collected daily and stored at 4°C, and the egg yolk was separated and stored at -30°C. Sera and eggs from non-immunized hens were treated in the same manner as mentioned above and used as controls.

Purification of IgY

Yolks from eggs obtained from 4 to 6 weeks after the initial immunization and those from non-immunized eggs were pooled separately. IgY was then isolated from these eggs using λ-carrageenan, according to the method described by Hatta et al (20). The pooled yolk samples were diluted with 3 volumes of 0.5% NaCl solution and homogenized, after which the yolk homogenate was slowly poured into a half volume of 0.4% (w/v) λ-carrageenan

solution while stirring, then kept for 1 h at room temperature. After centrifugation at 10,000 ×*g* for 20 min at 4°C, precipitated lipoproteins were removed. The water-soluble fraction was further purified by salt precipitation by adding 15% sodium sulfate (w/v) and then centrifuged at 12,000 ×*g* for 30 min. The resulting precipitate was dissolved in 10 mM disodium hydrogen phosphate solution; this was repeated twice. The final precipitate was dissolved in 10 mM disodium hydrogen phosphate solution and dialyzed against 4 liters of the same solution 3 times at 5°C and then lyophilized. The purity of the resulting IgY was checked by gel filtration chromatography using a high-pressure liquid chromatography (HPLC) system equipped with a TSK gel G3000SW column (TOSOH), with samples eluted with 0.1 M of potassium phosphate buffer containing 0.2 M of NaCl (pH 7.2) at a flow rate of 0.5 ml/min and detected by the measurement of absorbance at 280 nm.

Western blot analysis

The 122k-HagA was run on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrically onto a nitrocellulose membrane. The membrane was divided into 3 parts and soaked in blocking buffer (5% non-fat dry milk in TBS-T) for 30 min at room temperature, then incubated with immunized or non-immunized IgY (600 μg in 6 ml of blocking buffer for each) for 1 h. After washing 4 times with TBS-T, the membranes were incubated with peroxidase-conjugated rabbit anti-chicken IgY or goat anti-rabbit IgG (1,000-fold dilution in blocking buffer for each) at room temperature for 1 h. Immuno-reactivity was detected by incubating the membranes with 0.02% (W/V) 4-methoxy-1-naphthol in TBS and 0.02% (V/V) H_2O_2.

Slot-blot assay

A synthetic peptide "APVQNLTGSSVG" (peptide-437AP), spanning the 437 to 448 amino acid stretch of 130k-HMGD (6), and r130k-HMGD were slot-blotted onto the PVDF membrane and probed with immunized or non-immunized IgY. The blots were visualized by incubation with a peroxidase-conjugated secondary antibody in the same manner described for the Western blot analysis, except for color detection using an ECL-Plus system (Amersham Biosciences Co., Tokyo, Japan).

Hemagglutinating assay

The hemagglutinating activity of the *P. gingivalis* cells was assayed using rabbit erythrocytes in round-bottomed microtiter plates. Fifty microlitres of *P. gingivalis* vesicles were transferred into microtiter wells and incubated with the indicated amount of antibody for 30 min at 37°C, after which 50 μl of 2% (v/v) rabbit erythrocytes were added and incubated for 1.5 h at 37°C.

Results

The 122k-HagA was highly purified to a single band in SDS-PAGE as shown in Fig. 2. Hens were then immunized with the purified immunogen. The egg yolks obtained 4 weeks after the first immunization, when the antibody titer had reached the highest level, were pooled and the IgY was purified using a λ-carrageenan method. The purity of the IgY was examined by gel filtration chromatography using an HPLC system and a single major peak (Fig. 3). The IgY from non-immunized eggs was also

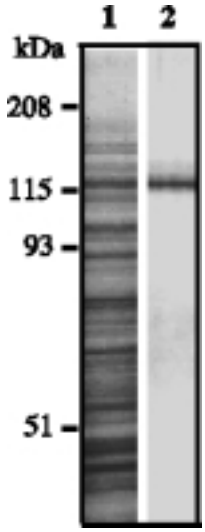

Fig. 2 SDS-PAGE analysis of purified r122k-HagA. Cell extract of *E. coli* transformant and the purified r122k-HagA were run on SDS-PAGE and stained with Coomassie Brilliant Blue R-250. 1, Cell extract of pMD160 transformant; 2, the purified fraction.

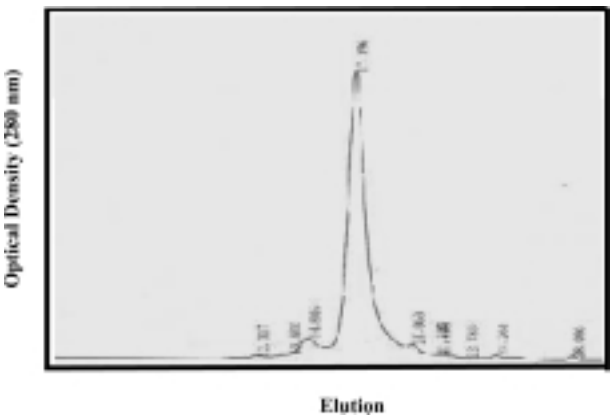

Fig. 3 Elution profile of the purified IgY fraction from r122k-HagA-immunized egg yolk using a gel filtration-HPLC column as described in the Materials and Methods.

purified by the same procedure and showed nearly the same elution profile with a single major peak using the HPLC system. The purity of the IgY from both immunized and non-immunized egg yolk samples was estimated to be over 90% of the total purified proteins, based on the protein elution patterns.

As shown in Fig. 4, the IgY against r122k-HagA reacted with r122k-HagA; in contrast, IgY from non-immunized hens did not react by Western blot analysis. Since PVQNLT in 130k-HMGD is suspected to be the functional domain of hemagglutinin (9), peptide-437AP containing this sequence was synthesized and slot-blotted onto a PVDF membrane, in order to examine whether anti-122k-HagA IgY could recognize the peptide-437AP. As shown in Fig. 5, the IgY clearly recognized r130k-HMGD as well as the peptide-437AP, suggesting that IgY reacted with the same epitope found in HagA and 130k-HMGD.

Finally, in order to confirm anti-122k-HagA IgY's neutralization of the hemagglutinating activity of *P. gingivalis*, the IgY was treated with *P. gingivalis* vesicles and examined. As shown in Fig. 6, the IgY significantly inhibited the vesicle-induced hemagglutinating activity in a dose-dependent manner.

Discussion

In periodontal disease, since the primary pathological sites are restricted to the surfaces of teeth and gingival tissue, passive immunization of tissues in the oral cavity can be achieved by a single mouth-rinse or local treatment of the gingival area. In a previous passive immunization approach, a monoclonal antibody that recognized the hem-agglutinating protease of *P. gingivalis* was applied to subgingival pockets in patients with periodontitis. Recolonization of *P. gingivalis* was significantly reduced for up to 9 months (21). However, a large amount of passive antibody is required for practical use. As IgY has the advantages of biosafety, cost-effectiveness, and the ability to produce large-scale antibody preparations without killing animals, it has recently attracted considerable attention as an alternative source of antibodies (18).

Adherence to host cells is required for virulence of mucosal pathogens; therefore, preventing the adherence of a particular bacterial pathogen by specific antibodies against the adhesin should prevent colonization and disease (22). It is noteworthy that when the *P. gingivalis* gene library was screened for clones that bind human oral epithelial cells, all positive gene clones had homology with *hagA* (23). RgpA-Kgp, carring PVQNLT motif, was recently found to be a potentially useful immunogen that elicits a protective immune response against subsequent colonization by *P. gingivalis* (24). Furthermore, an antibody against the *P. gingivalis* 130k-HMGD inhibited hemolysis activity of *P. gingivalis* cells (25). Taken together with the results of this study, these data suggest that 122k-HagA might be useful for developing a protective vaccine against periodontal diseases associated with *P. gingivalis*.

In this study, a novel IgY established by immunization of 122k-HagA significantly inhibited hemagglutinating activity (Fig. 6). The functional motif PVQNLT is known to be encoded in several hemagglutinin molecules, as well as in hemagglutinin-related proteases in many *P. gingivalis* strains (9). Our immunological analyses using slot blotting demonstrated that anti-122k-HagA IgY reacted with 122k-HagA and the functional domain peptide-437AP (Fig. 5). These findings suggest that our novel IgY neutralized

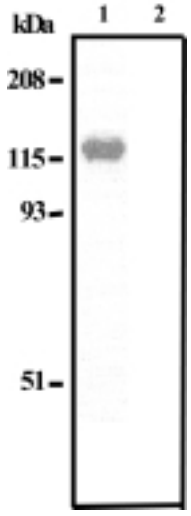

Fig. 4 Western blot analysis of the immunoreactivity of r122k-HagA-immunized IgY. The purified r122k-HagA was run on SDS-PAGE and transferred onto nitrocellulose filter and probed with r122k-HagA-immunized (1) and non-immunized IgY (2) as a control.

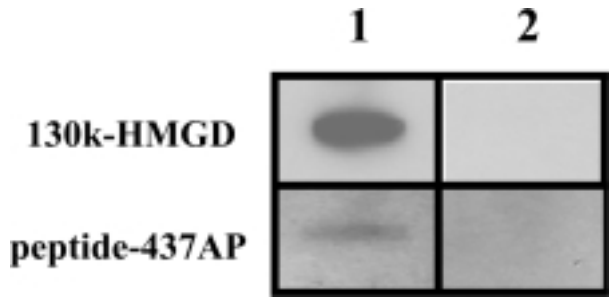

Fig. 5 Slot-blot analysis of the immunoreactivity of r122k-HagA-immunized IgY. The r130k-HMGD (0.2 ng) and peptide-437AP (12.2 μg) was slot blotted onto nitrocellulose filter and probed with r122k-HagA-immunized IgY (1) and non-immunized IgY (2) as a control.

hemagglutinating activity through binding the functional domain PVQNLT, suggesting that our IgY has a large capacity to neutralize various hemagglutinating molecules harboring the PVQNLT motif.

When considering passive immunization against periodontal diseases, the pathological focus should be on gingival tissues. Application of a specific antibody that neutralizes bacterial adherence can provide practical and satisfactory treatment. The immunological properties of IgY are somewhat different from those of mammalian IgG. IgY does not associate with mammalian complements and the binding activity of IgY with the Fc receptor on cell surface is much lower than that of IgG (26). Thus, IgY is not anticipated to have the cell killing activity of the complement system and opsonization. However, such cell killing activities sometimes lead to unwanted immunoresponses such as allergic reactions. When considering the safety of passive antibodies for neutralizing virulent factors, IgY may avoid the unwanted cellular immune response and therefore be a favorable component for passive immuntherapy. Moreover, chicken eggs are a cheap and convenient source for mass production of IgY (27). IgY has been used extensively for treatment and prevention of various infections in animals and humans with a high degree of success (28). Recently, we established IgY capable of inhibiting the hemagglutinating activity of *P. gingivalis* using r130k-HMGD (29); however, r130k-HMGD was difficult to recover and purify from *E. coli* clone inclusion bodies. In contrast, 122k-HagA can be isolated as a soluble form and purified with higher efficiency from the gene clone cell extracts. Since the IgY against 122k-HagA recognized both r130k-HMGD and the functional domain peptide, 122k-HagA appears to be a useful and favorable immunogen for IgY production.

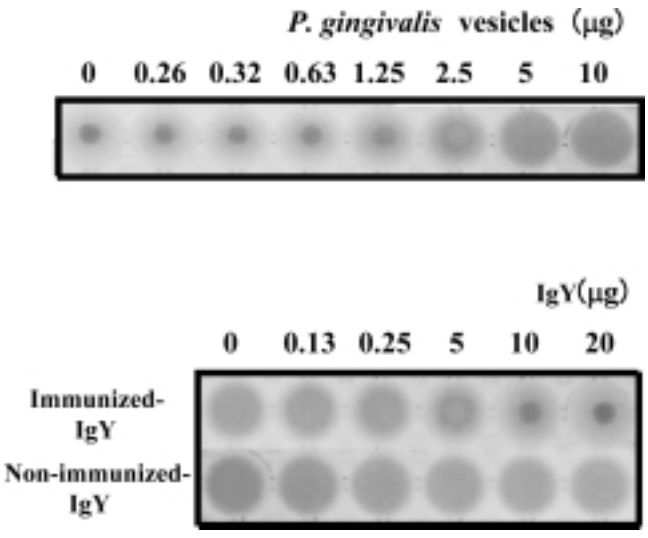

Fig. 6 Inhibition of hemagglutinating activity of *P. gingivalis* vesicles by r122k-HagA-immunized IgY. The optimum amount of *P. gingivalis* vesicles was determined to be 5 ng/50 μl by titration of hemagglutinating activity (A). Effect of various concentrations of r122k-HagA-immunized IgY on hemagglutinating activity was examined using 5 ng of *P. gingivalis* vesicles.

In conclusion, the IgY against 122k-HagA presented herein could be useful in the development of passive immunization as well as in assessing the treatment of periodontal diseases caused by *P. gingivalis* infection.

Acknowledgments

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