Production of IgY Against a *Porphyromonas gingivalis* 40-kDa Outer Membrane Protein That Inhibits Its Hemagglutinating Activity

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Abstract

Porphyromonas gingivalis has been implicated as an important pathogen in periodontitis. Hemagglutinins have been identified in the adhesion of *P. gingivalis* onto the gingival tissue cells and the attachment and lysis of erythrocytes to facilitate the uptake of iron, an essential nutrient for *P. gingivalis*. A 40-kDa outer membrane protein (OMP) gene has been cloned from *P. gingivalis*. Because the monoclonal antibody against recombinant (r) 40-kDa OMP inhibited the hemagglutinating activity and the polymeric form of the recombinant protein expressed hemagglutinating activity, the 40-kDa OMP is thought to be a hemagglutinin. Moreover, the immunodominant domain has been identified as "WPRVGQLFIALDQTLGIPGF", designated as peptide C, using phage display epitome mapping. On the other hand, chicken yolk antibody (IgY) has been shown to prevent bacterial infection and thought to be a useful and safe antibody for passive immunotherapy. In this study, r40-kDa OMP was highly purified and hens were inoculated with it to produce IgY. The purified IgY reacted with r40-kDa OMP and the synthetic peptide C, and significantly inhibited the hemagglutinating activity of *P. gingivalis*. Thus, IgY may be useful in the development of passive immunization against periodontal diseases caused by *P. gingivalis* infection.

Keywords :

periodontal diseases, IgY, *Porphyromonas gingivalis*, outer membrane protein, hemagglutinin

Introduction

Porphyromonas gingivalis has been implicated as an important pathogen in the development of periodontitis (1, 2). Adherence of bacteria to host tissue cells is a crucial step and a causative factor in pathogenesis. Hemagglutinins have been suggested to play a role in the initial adherence of the bacteria to the host tissues (3). It has been reported that *P. gingivalis* has a high adherence activity to erythrocytes and epithelial cells (4) and hemagglutinins have been identified as playing an important role in adhesion (3). Moreover, hemin is essential for the growth of *P. gingivalis* (5) and it is derived from erythrocytes in the natural niche of the organism. Thus, the hemagglutinin molecule may be particularly important for this microorganism, not only for adherence to the gingival tissue cells but also for the

attachment and lysis of erythrocytes for hemin uptake.

We previously succeeded in the molecular cloning of a 40-kDa outer membrane protein (OMP) from *P. gingivalis* 381 by immunological screening using antibody against an outer membrane preparation (6) and found the OMP acted as a coaggregation factor to oral bacteria (7). Although recombinant (r) 40-kDa OMP did not expressed coaggregation and hemagglutination, the polymeric form of r40-kDa OMP expressed coaggregation (8) and hemagglutination (9). Moreover, a monoclonal antibody was generated that exhibited highly inhibitory activity against hemagglutination of the polymeric form of r40-kDa OMP and *P. gingivalis* vesicles. Furthermore, the immunodominant domain was identified as "WPRVGQLFIALDQTLGIPGF" by phage display

epitome mapping (10).

In the development of a passive immunotherapy, the biosafety of the antibody is a vital prerequisite and passive immunization approaches against oral infectious diseases have been developed with such safety in mind (11). Passive immunization therapies generally require a large amount of the antibody preparation because of the oral administration route utilized.

Recently, a specific antibody from hen egg yolk preparations (IgY) has attracted considerable attention and has been studied extensively with oral and gastrointestinal pathogens in both humans and animals. Results have shown the advantages of IgY, including reduced cost, biosafety, and easy preparation in large quantities from the eggs of immunized hens (12).

In the present study, to develop a passive immunotherapy against periodontal diseases, we constructed a specific IgY against r40-kDa OMP and examined its ability to inhibit *P. gingivalis* hemagglutinating activity.

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% N₂, 10% H₂, and 10% CO₂ and the vesicle fraction was prepared as previously reported (13).

Production of IgY

Recombinant 40-kDa OMP was prepared by a method reported previously (14). Rhode Island Red hens (200 days old) were intramuscularly immunized in both legs with purified r40-kDa OMP (0.25 mg/ml/animal) using Freund's complete adjuvant (Becton Dickinson, NJ, USA). Following the initial immunization, two booster injections were given 2 weeks apart using Freund's incomplete adjuvant. A 1-ml sample of blood was collected from each animal from the vein below the wing every 2 weeks follow-

ing 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 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.* (15). The pooled yolk samples were diluted with three 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 with the addition of 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 L of the same solution three times at 5 °C and then lyophilized. The purity of the resulting IgY was checked by gel filtration chromatography using a TSK gel G3000SW column (TOSOH Corp., Tokyo, Japan); samples were 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 IgY was detected by the measurement of absorbance at 280 nm.

Western blot analysis and dot-blot assay

The r40-kDa OMP was isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrically onto a nitrocellulose membrane. The membrane was soaked in blocking buffer [5% non-fat dry milk in Tris-buffered saline with Tween 20 (TBS-T)] for 30 min at room temperature, then

incubated with immunized or non-immunized IgY (600 μ g in 6 ml of blocking buffer for each) for 1 h. After washing four 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. Immunoreactivity was detected by incubating the membranes with 0.02% (w/v) 4-methoxy-1-naphthol in Tris-buffered saline and 0.02% (v/v) H_2O_2 .

The synthetic peptide “WPRVGQLFIALDQTLGIPGF” as a functional domain of r40-kDa OMP (15) was dot-blotted onto a polyvinylidene fluoride 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., Buckinghamshire, England).

Hemagglutinating assay

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

Results

The r40-kDa OMP was highly purified to a single band by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). Hens were then immunized with the purified r40-kDa OMP. The egg yolks obtained 6 weeks after the first immunization showed that was when the antibody titer had reached the high level (Fig. 1). IgY was purified using the λ -carrageenan method and the purity of the IgY was examined by gel filtration chromatography using a high performance liquid chromatography system (Fig. 2). The IgY from non-immunized eggs was also purified by the same procedure. The purity of the

Fig. 1. Antibody titer in serum (A) and egg yolk (B) from hens immunized with r40-kDa OMP (closed circles) and non-immunized hens (open circles).

IgY from both immunized and non-immunized egg yolk samples was estimated to be over 92% of the total purified proteins, based on the protein elution patterns.

As shown in Figure 3, the IgY against r40-kDa OMP reacted with r40-kDa OMP; in contrast, IgY from non-immunized hens was not recognized in western blot analysis.

Because “WPRVGQLFIALDQTLGIPGF” in r40-kDa OMP is suspected to be the functional domain of

Fig. 2. Elution profile of purified IgY from r40-kDa OMP-immunized eggs using a gel filtration-high performance liquid chromatography system. Elution and detection of proteins were carried out as described in the Materials and Methods section.

Fig. 3. Western blot analysis of immunoreactivity of IgY. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a protein molecular weight marker (S) and r40-kDa OMP (1) stained with Coomassie brilliant blue R-250. After proteins were transferred to a nitrocellulose membrane probed with rabbit polyclonal antibody against r40-kDa OMP (2). Non-immunized IgY (3) and immunized IgY against r40-kDa OMP (4).

the hemagglutinin (Fig. 4A), a synthetic peptide containing this sequence was dot-blotted onto a polyvinylidene fluoride membrane and probed by anti-r40-kDa OMP IgY. As shown in Figure 4B, the IgY clearly recognized r40-kDa OMP as well as the peptide “WPRVGQLFIALDQTLGIPGF”.

Fig. 4. Dot-blot analysis of the immunoreactivity of IgY. A. Amino sequence of 40-kDa OMP. Box shows peptide C. B, r40-kDa OMP and peptide C were dot-blotted onto a polyvinylidene fluoride membrane and reacted with non-immunized IgY (1) and r40-kDa OMP-immunized IgY (2).

Fig. 5. Effect of IgY on hemagglutinating activity of *P. gingivalis* vesicles. The optimum amount of the vesicles suspension used in the hemagglutination assay was determined to be 2.5 ng/50 μ l. The assay of hemagglutinating activity was performed as described in the Materials and Methods section.

Finally, to confirm the inhibitory ability of anti-r40-kDa OMP IgY against the hemagglutinating activity of *P. gingivalis*, the IgY was treated with *P. gingivalis* vesicles and examined. As shown in Figure 5, the IgY significantly inhibited *P. gingivalis* vesicle-induced hemagglutinating activity in a dose-dependent manner.

Discussion

On the way to examine the pathogenic role of the 40-kDa OMP, we found that although the r40-kDa OMP itself did not express hemagglutinating activ-

ity, the polymeric r40-kDa OMP using cross-linking agents expressed the hemagglutinating activity (8). This finding demonstrated that the 40-kDa OMP is one of the hemagglutinins of *P. gingivalis*. We also constructed several monoclonal antibodies against r40-kDa OMP and identified a monoclonal antibody that significantly inhibited the hemagglutinating activity of *P. gingivalis* (9). Furthermore, the immunodominant domain recognized by the monoclonal antibody was identified as "WPRVGQLFIALDQTLGIPGF" by phage display epitope mapping (10), suggesting that this motif may be a functional domain expressing hemagglutinating activity.

Adhesion of bacteria to host tissues is a prerequisite for colonization and an important factor in bacterial pathogenesis. Colonization of *P. gingivalis* in the subgingival area is critical in the pathogenic process of periodontal disease. Hemagglutinins have been suggested to play a role in the initial adherence of the bacteria to erythrocytes and in the uptake of iron ions that are essential for the growth of *P. gingivalis*.

Toward the development of passive immunotherapy, the production of safe and useful antibodies is a significant issue. In the consideration of 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.

In this study, we purified r40-kDa OMP and inoculated hens with it to produce IgY against 40-kDa OMP. The purified IgY reacted with r40-kDa OMP and the functional domain peptide "WPRVGQLFIALDQTLGIPGF". Moreover, IgY significantly inhibited the hemagglutinating activities of *P. gingivalis* vesicles.

Although whole-cell vaccines are effective at preventing bacterial infectious diseases, certain vaccines have been associated with unwanted immunoreponse. A safe passive antibody that can neutralize hemagglutinin is important in immunotherapy. In periodontal disease, because 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.

Recently, it has been recognized that oral infection, especially periodontitis, may affect the course and pathogenesis of a number of systemic diseases, such as cardiovascular disease, bacterial pneumonia, diabetes mellitus, and low birth weight (16, 17). Booth *et al.* (18) reported that the administration of monoclonal antibodies against *P. gingivalis* to periodontal pockets of patients significantly reduced the recolonization of *P. gingivalis* for up to 9 months. Thus, the passive immunization approach may be made possible for practical use against systemic diseases through the prevention of periodontitis. Although this concept is meaningful because the effect of periodontal diseases on systemic diseases has been recognized in the dental society, the general society does not consider periodontal diseases to be especially serious. Therefore, to promote a practical vaccine for humans, the vaccine must be absolutely safe. In addition, a large amount of passive antibody is also required for the realization of practical use. [Please clarify the previous statement. Why is the large amount necessary? Is it because of possible gastric degradation as the result of oral administration, or some other reason?]

Because IgY has the advantages of biosafety, cost-effectiveness, and the ability to be produced in large-scale antibody preparations without killing animals, it has recently attracted considerable attention as an alternative source of antibodies. 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 surfaces is much lower than that of IgG (19). Thus, IgY is not anticipated to have the cytotoxicity of the complement system. Such cytotoxicity may 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 im-

munotherapy. Moreover, chicken eggs are an inexpensive and convenient source for mass production of IgY for enteric infections (20). IgY has been used extensively for the treatment and prevention of dental caries with a high degree of success (21). More recently, Yokoyama *et al.* (22) succeeded in producing IgY against *P. gingivalis* gingipains that play an important role in the progression of periodontitis (23). They found that IgY was capable of inhibiting gingipain activity.

Furthermore, they applied the IgY subgingivally in patients with periodontitis who harbored *P. gingivalis* in their subgingival flora and demonstrated a significant reduction in the levels of *P. gingivalis* (24). These findings suggest that passive immunotherapy using IgY is effective for preventing the colonization of *P. gingivalis* and that IgY is a useful agent for treating *P. gingivalis* infection.

Because 40-kDa OMP is an important molecule for hemagglutination, passive immunotherapy using IgY against 40-kDa OMP may be emphasized as an approach to preventing periodontal disease, and subsequent systemic disease, caused by *P. gingivalis*. Further studies focusing on the potential role of IgY in preventing infections of *P. gingivalis* are underway.

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References

1. Slots J, Genco RJ: Black-pigmented *Bacteroides* species, *Capnocytophaga* species, and *Actinobacillus actinomycetemcomitans* in human periodontal disease: virulence factors in colonization, survival, and tissue destruction. J Dent Res, 63: 412-421, 1984.
2. Holt SC, Kesavalu L, Walker S, Genco CA: Virulence factors of *Porphyromonas gingivalis*. Periodontol 2000, 20: 168-238, 1999.
3. Lamont RJ, Jenkinson HF: Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. Microbiol Mol Biol Rev, 62: 1244-1263, 1998.
4. Takazoe I, Nakamura T, Okuda K: Colonization of the subgingival area by *Bacteroides gingivalis*. J Dent Res, 63: 422-426, 1984.
5. Martinex JL, Delgado-Iribarren A, Baquero F: Mechanisms of iron acquisition and bacterial virulence. FEMS Microbiol Rev, 75: 45-56, 1990.
6. Abiko Y, Hayakawa M, Aoki H, Kikuchi T, Shimatake H, Takiguchi H: Cloning of a *Bacteroides gingivalis* outer membrane protein gene in *Escherichia coli*. Arch Oral Biol, 35: 689-695, 1990.
7. Hiratsuka K, Abiko Y, Hayakawa M, Ito T, Sasahara H, Takiguchi H: Role of *Porphyromonas gingivalis* 40-kDa outer membrane protein in the aggregation of *P. gingivalis* vesicles and *Actinomyces viscosus*. Arch Oral Biol, 37: 717-724, 1992.
8. Nonaka E, Kiyama-Kishikawa M, Abiko Y: Identification of 40-kDa outer membrane protein as an aggregation factor of *Porphyromonas gingivalis* to *Streptococcus gordonii*. J Oral Sci, 43: 239-243, 2001.
9. Abiko Y, Hiratsuka K, Kiyama-Kishikawa M, Hayakawa M, Tagawa H, Lin M-C, Lee Y-S: Induction of hemagglutination by recombinant *Porphyromonas gingivalis* 40-kDa outer membrane protein. Int J Oral-Med Sci, 2: 54-56, 2004.
10. Chang W-J, Kishikawa-Kiyama M, Shibata Y, Lee S-Y, Abiko Y: Inhibition of *Porphyromonas gingivalis* hemagglutinating activity by synthetic peptides derived from phage display selection using monoclonal antibody against the recombinant outer membrane protein. Hybrid Hybridomics, 23: 352-356, 2004.
11. Abiko Y: Passive immunization against dental caries and periodontal disease: development of recombinant and human monoclonal antibodies. Crit Rev Oral Biol Med, 11: 140-153, 2000.
12. Hatta H, Ozeki M, Tsuda K: Egg yolk antibody IgY and its application, In: Hen Eggs Their Basic and Applied Science, Yamamoto T, Juneja LR, Hatta H, Kim M eds, CRC Press, pp151-178, 1977.
13. Grenier D, Mayrand D: Functional characterization of extracellular vesicles produced by *Bacteroides gingivalis*. Infect Immun, 55: 111-117, 1987.
14. Kawamoto Y, Hayakawa M, Abiko Y: Purification and immunochemical characterization of a recombinant outer membrane protein from *Bacteroides gingivalis*. Int J Biochem, 23: 1053-1061, 1991.
15. Hatta H, Kim M, Yamamoto T: A novel isolation method for hen egg yolk antibody, "IgY". Agric Biol Chem, 54: 2531-2535, 1990.
16. Li X, Kolltveit KM, Tronstad L, Olsen I: Systemic diseases caused by oral infection. Clin Microbiol Rev, 13: 547-558, 2000.

17. Moutsopoulos NM, Madianos PN : Low-grade inflammation in chronic infectious diseases: paradigm of periodontal infections. Ann N Y Acad Sci, 1088 : 251-264, 2006.
18. Booth V, Ashley FP, Lehner T : Passive immunization with monoclonal antibodies against *Porphyromonas gingivalis* in patients with periodontitis. Infect Immun, 64 : 422-427, 1996.
19. Gardner PS, Kaye S : Egg globulins in rapid virus diagnosis. J Virol Methods, 4 : 257-262, 1982.
20. Carlander D, Kollberg H, Wejaker PE, Larsson A : Personal immunotherapy with yolk antibodies for the prevention and treatment of enteric infections. Immunol Res, 21 : 1-6, 2001.
21. Smith DJ, King WF, Godiska R : Passive transfer of immunoglobulin Y antibody to *Streptococcus mutans* glucan binding protein B can confer protection against experimental dental caries. Infect Immun, 69 : 3135-3142, 2001.
22. Yokoyama K, Sugano N, Rahman AK, Oshikawa M, Ito K : Activity of anti- *Porphyromonas gingivalis* egg yolk antibody against gingipains in vitro. Oral Microbiol Immunol, 22 : 352-355, 2007.
23. Imamura Ti : The role of gingipains in the pathogenesis of periodontal disease. J Periodontol, 74 : 111-118, 2003.
24. Yokoyama K, Sugano N, Shimada T, Shofiqur RA, Ibrahim el-SM, Isoda R, Umeda K, Sa NV, Kodama Y, Ito K : Effects of egg yolk antibody against *Porphyromonas gingivalis* gingipains in periodontitis patients. J Oral Sci, 49 : 201-220, 2007.