Specific Antibodies to *Porphyromonas gingivalis* Lys-Gingipain by DNA Vaccination Inhibit Bacterial Binding to Hemoglobin and Protect Mice from Infection

MASAE KUBONIWA,[1,2] ATSUO AMANO,[3,4]* SATOSHI SHIZUKUISHI,[2] ICHIRO NAKAGAWA,[1]
AND SHIGEYUKI HAMADA[1]

*Departments of Oral Microbiology,[1] Preventive Dentistry,[2] and Oral Science Methodology[3] and Division of
Special Care Dentistry,[4] Osaka University Graduate School of Dentistry, Suita-Osaka, Japan*

Lys-gingipain (KGP), a lysine-specific cysteine proteinase, is one of the major virulence factors of *Porphyromonas gingivalis*. Here we examined the involvement of the catalytic domain of KGP (KGP_{cd}) in hemoglobin binding by *P. gingivalis*, using a specific immunoglobulin G (IgG) elicited by the administration of plasmid DNA encoding KGP_{cd} or the catalytic domain of Arg-gingipain (RGP_{cd}). The pSeq2A/kgp_{cd} and pSeq2B/rgp_{cd} plasmids were constructed by the ligation of kgp_{cd} and rgp_{cd} DNA fragments, respectively. Female BALB/c mice were immunized with each of these plasmids. pSeq2A/kgp_{cd} elicited a strong response to recombinant KGP_{cd} ($rKGP_{cd}$), as well as to comparably produced $rRGP_{cd}$-reactive antibodies. The serum antibodies elicited by pSecTag2B/rgp_{cd} also cross-reacted with $rKGP_{cd}$ as well as $rRGP_{cd}$. Anti-KGP_{cd} IgG significantly inhibited hemoglobin binding by *P. gingivalis*. Furthermore, the inhibition of hemoglobin binding was markedly enhanced by a combination of anti-KGP_{cd} and anti-fimbriae. Anti-RGP_{cd} IgG showed a negligible inhibitory effect, while both anti-KGP_{cd} and anti-RGP_{cd} IgGs showed significant inhibitory effects on Lys- and Arg-specific proteolytic activities and on the growth of *P. gingivalis* under iron-restricted conditions where supplemented hemoglobin was the sole iron source. Immunized mice were challenged by intraperitoneal inoculation with *P. gingivalis*. All nonimmunized mice died within 72 h; however, vaccination with pSeq2A/kgp_{cd} and pSeq2B/rgp_{cd} prevented inflammatory responses and prolonged the survival rate of immunized mice by 43 and 27%, respectively. These results suggest that KGP_{cd} acts as a hemoglobin-binding protein and can also be useful as an immunogen inducing a protective response to *P. gingivalis* infection.

Iron is an essential nutrient for most living organisms and is abundant in the human body. Free iron, however, is kept at an extremely low level, far below that needed for bacterial growth, resulting in a limitation of bacterial infection (25, 44). Iron is bound extracellularly to transferrin and lactoferrin and is contained intracellularly within ferritin, hemosiderin, and such hemin-containing compounds as hemoglobin and myoglobin (25). Many aerobic and facultative anaerobic bacteria have developed a specific iron acquisition system by using siderophores, which are low-molecular-mass iron chelators that remove iron that has been complexed to host iron-carrying proteins (8). However, some bacterial genera can use heme, hemoglobin, transferrin, lactoferrin, and hemopexin iron directly without the involvement of siderophores (16, 32, 46).

Periodontal diseases are infectious and induce inflammation in the supportive tissues of teeth in response to the accumulation of pathogens in the subgingival crevice (24, 45). The black-pigmented obligate anaerobe *Porphyromonas gingivalis* is considered to be the most important agent of these infections and causes several types of periodontal diseases, including adult periodontitis (15, 24, 45). The availability of iron in gingival crevicular fluid is crucial for the growth and virulence of this organism, which produces no siderophore (5). *P. gingi-*

valis can utilize hemin as an iron source and also seems to store hemin on its cell surface, which causes the black pigmentation of its colonies (36). *P. gingivalis* specifically utilizes several hemin-containing compounds as iron sources (5, 14); of these, hemoglobin supports bacterial growth much more efficiently than do transferrin, hemin, or inorganic iron compounds (38). We previously reported that the 51-kDa catalytic domain of *P. gingivalis* Lys-gingipain, lysine-specific cysteine proteinase (KGP_{cd}, encoded by kgp_{cd}), has significant binding ability to human hemoglobin (specific association constant, $K_a = 6.90 \times 10^7$) and that the recombinant polypeptide of KGP_{cd} also has both a hemoglobin-binding activity and a significant inhibitory effect against the binding of whole-cell extracts to hemoglobin (20). It has been also reported that deletion of the *P. gingivalis* kgp_{cd} gene generates mutants without pigmentation (6, 7, 23, 30, 40). These findings strongly suggest that KGP_{cd} plays a critical role in hemin acquisition within the cells. On the other hand, HGP15, which has a deduced molecular size of 15 kDa and is encoded by *hgp15* downstream of kgp_{cd} and rgp_{cd} (catalytic domain of Arg-gingipain [RGP]-encoding gene) and within *hagA* (hemagglutinin-encoding gene), was shown to have a marked affinity for hemoglobin ($K_a = 2.04 \times 10^7$) (27). It was also shown that *P. gingivalis* fimbriae strongly bind to hemoglobin ($K_a = 2.43 \times 10^6$) (2); however, the fimbriae were demonstrated to have no association with hemin accumulation and storage by *P. gingivalis* (6). The above findings concluded that KGP_{cd} and/or HGP15 may play important roles for hemin utilization from hemoglobin of *P. gingivalis*; however, the exact

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* Corresponding author. Mailing address: Department of Oral Science Methodology, Osaka University Graduate School of Dentistry, 1-8 Yamadaoka, Suita-Osaka 565-0871, Japan. Phone: 81-6-6879-2283. Fax: 81-6-6879-2976. E-mail: amanoa@dent.osaka-u.ac.jp.
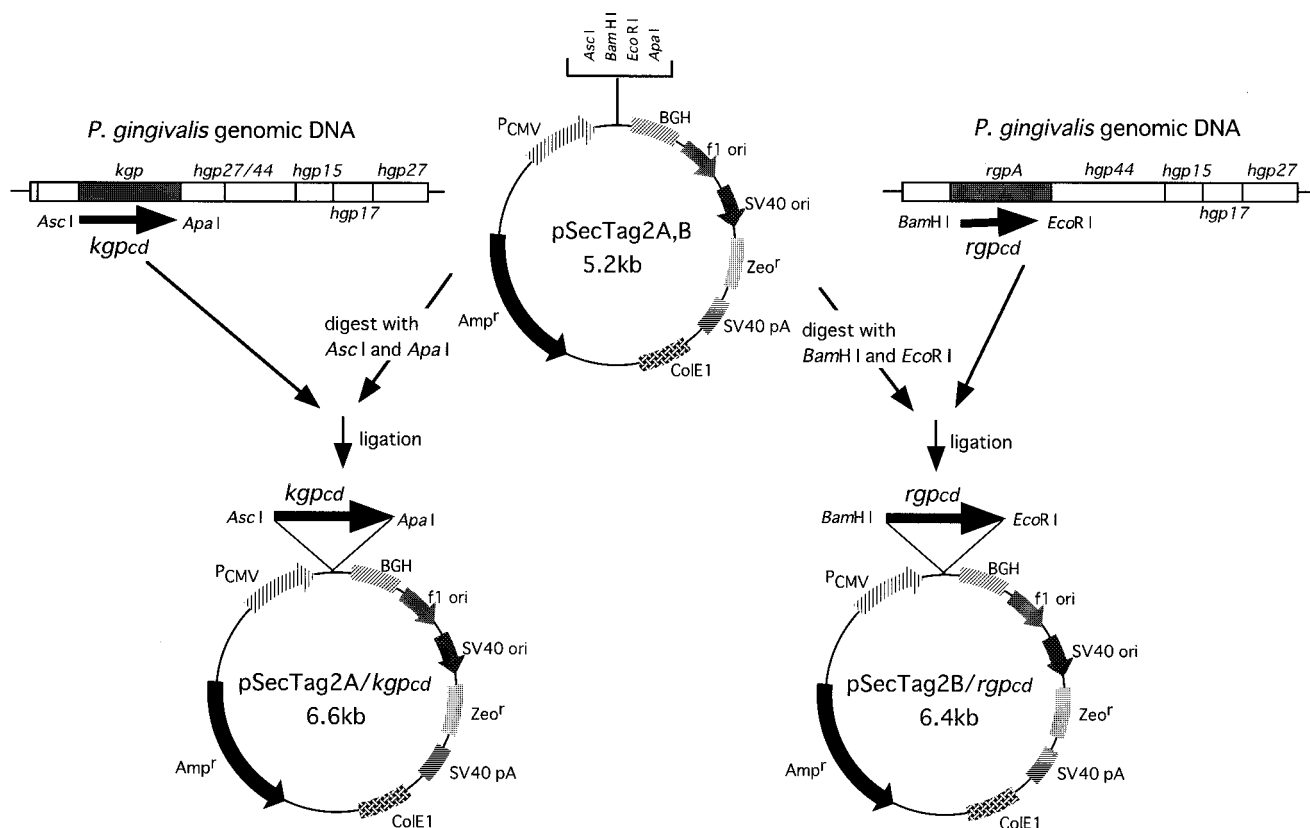
FIG. 1. Construction of plasmid DNA for immunization. Amplified *kgp*$_{cd}$ and *rgp*$_{cd}$ genes were inserted into the eukaryotic expression vectors pSecTag2A and pSecTag2B, respectively (see Materials and Methods). SV40, simian virus 40; P$_{CMV}$, human cytomegalovirus immediate-early promoter; BGH, bovine growth hormone gene (provides the polyadenylation [pA] signal); Amp^r, ampicillin resistance gene; Zeo^r, Zeocin resistance gene.

roles of these two molecules in hemin-hemoglobin transport in *P. gingivalis* remain to be defined.

Antigen-encoding plasmid DNA immunization (DNA vaccine) is considered to be a powerful approach to the generation of needed antigenic proteins by the host cells. This novel strategy can induce cellular and humoral immune responses to a variety of pathogens, including viruses, parasites, bacteria (35), and tumor cells (19). The antibody responses induced by DNA vaccinations were reportedly lower than those induced by classical immunizations of antigens with adjuvants, because of the low level of secretion of the expressed antigens from the transfected cells (10, 41). Recently, plasmid vectors with a strong heterogenous signal sequence, which mediates efficient antigen secretion in vivo, have been shown to induce significantly higher antibody levels than did previous vectors for little-secreted antigens (10, 41). Since plasmid DNA immunization can be used for immunization of the host without purification of antigenic proteins, this strategy is considered useful for eliciting specific antisera in experimental animals (10, 41). Recently, it was shown that humoral responses were effectively induced against *P. gingivalis* fimbriae by a DNA vaccination using a *P. gingivalis* fimbrillin-coding plasmid (18); however, no other studies with this organism have been reported.

We examined the role of the KGP$_{cd}$ in hemoglobin binding by *P. gingivalis* using specific immunoglobulins elicited by plasmid DNA encoding KGP$_{cd}$ or RGP$_{cd}$. These plasmids were

constructed with a signal sequence to secrete antigens for effective antibody responses. Furthermore, the constructed DNA vaccines were used in a genetic immunization strategy against *P. gingivalis* challenge in a murine model to evaluate the effect of the KGP$_{cd}$ as an immunogen.

MATERIALS AND METHODS

Bacteria. *P. gingivalis* strains ATCC 33277 and W50 were grown in Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, Md.) supplemented with yeast extract (1 mg/ml), menadione (1 µg/ml), and hemin (5 µg/ml) in an anaerobic chamber, as described previously (4). Bacterial cells were harvested, washed in prereduced sterile phosphate-buffered saline (PBS; 10 mM phosphate buffer containing 0.15 M sodium chloride [pH 7.4]), and resuspended in the same buffer. The number of bacteria in the suspension was estimated by measuring the optical density at 600 nm and extrapolated from a standard curve, as described previously (26). To prepare the bacterial extracts, washed cells were suspended in ice-cold PBS containing 3% (wt/vol) zwitterionic detergent 3-[(3-cholamido-propyl)-dimethylammonio]-1-propanesulfonate (CHAPS; Pierce, Rockford, Ill.) followed by stirring at 4°C for 40 min. The supernatant obtained by centrifugation at 20,000 × *g* at 4°C for 1 h was thoroughly dialyzed against PBS and then used as the extract. *Escherichia coli* XL10-Gold (Stratagene, La Jolla, Calif.) was cultured in Luria-Bertani broth or medium containing 1.5% agar supplemented with ampicillin (100 µg/ml).

Construction of plasmid DNA for immunization. Antigen-encoding plasmids were constructed as illustrated in Fig. 1. *P. gingivalis* ATCC 33277 genomic DNA was prepared as described previously (3) and used to amplify the *kgp*$_{cd}$ gene encoding whole amino acid residues of mature KGPcd (489 amino acids (aa) and the *rgp*$_{cd}$ gene encoding the RGP$_{cd}$ polypeptide corresponding to aa 9 to 431 of whole mature molecule (423 residues), using a PCR method. The PCR primers used were as follows; for *kgp*$_{cd}$, the forward primer (5′-TAGGCGCGCCGATG
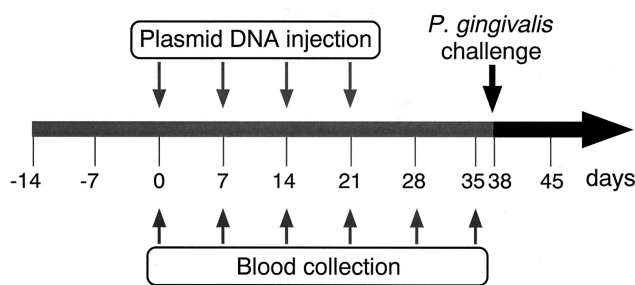
FIG. 2. Protocol for intramuscular immunizations with plasmid DNA containing kgp_{cd} or rgp_{cd} and intraperitoneal challenge in mice. Each plasmid (50 μg) was injected into the quadriceps muscles weekly for a total of four inoculations. At 38 days after the first immunization, mice were intraperitoneally challenged with *P. gingivalis* W50 (9×10^9 CFU), and their health status was observed over 14 days.

TTTATACAGATCATGGCGAC-3′) and the reverse primer (5′-TAGGGCCC ACGGGAAGCTTCTGCCTTCTTTGC-3′) incorporated *Asc*I and *Apa*I sites; and for rgp_{cd}, the forward primer (5′-TAGGATCCAATGGTCGTATGATCG TCATCG-3′) and the reverse primer (5′-GTGAATTCTCACACTTTCACATC CTTTATC-3′) incorporated *Bam*HI and *Eco*RI sites (Fig. 1). PCR was performed with a model PCR 2400 thermal cycler (Perkin-Elmer, Norwalk, Conn.), using parameters described previously (20). The resultant kgp_{cd} and rgp_{cd} fragments were inserted into the eukaryotic expression vectors pSecTag2A and pSecTag2B (Invitrogen, Groningen, The Netherlands), respectively. DNA sequencing was performed to confirm the correct in-frame coding alignments of the plasmids with a DNA sequencer (ABI PRISM 310 genetic analyzer; Perkin-Elmer), and the constructed pSecTag2A/kgp_{cd} and pSecTag2B/rgp_{cd} were used for plasmid DNA immunization.

Immunization and murine infection protocols. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the Osaka University Graduate School of Dentistry prior to the experiments. Female BALB/c mice (6 weeks old) were maintained in horizontal-flow cabinets and provided with sterile food and water ad libitum. A schematic of the experimental design is shown in Fig. 2. Briefly, mice were immunized by injection of each plasmid DNA dissolved at 1 mg/ml in 50 μl of sterile PBS into the quadriceps muscle. Control mice were immunized with 50 μl of intact pSecTag2A plasmid at 1 mg/ml or PBS. Starting 1 week after primary immunization, the mice were boosted three times at 1-week intervals with the same quantities of each DNA solution. At 0, 7, 14, 21, 28, and 35 days after the primary immunization, blood samples were collected from the orbital sinus or plexus of each mouse and sera were collected by centrifugation following clotting at 4°C. Antibodies elicited by pSecTag2A/kgp_{cd} and pSecTag2B/rgp_{cd} were used as anti-KGP_{cd} and anti-RGP_{cd} antibodies, respectively, for further studies. At 38 days after the first immunization, the mice were intraperitoneally infected with inoculation of 9×10^9 CFU of *P. gingivalis* W50. The animals were monitored for signs and symptoms of infection and evaluated for (i) the size of eroded skin lesions on the abdomens, (ii) cachexia, and (iii) death. Lesion size was expressed as the average maximum diameter achieved during the 2-week-postinfection period.

Preparation of $rKGP_{cd}$ and $rRGP_{cd}$ proteins. The recombinant KGP_{cd} polypeptide ($rKGP_{cd}$) was expressed in *Escherichia coli* BL21 (DE3) and purified as described previously (20). To generate the $rRGP_{cd}$ polypeptide, the rgp_{cd} fragments used for pSecTag2B/rgp_{cd} construction were inserted into pGEX2T (Amersham Pharmacia Biotech), which is the vector for expression of the glutathione *S*-transferase (GST) fusion protein. The construct was then transformed to *E. coli* XL-10GOLD as described previously (3). The expression of the GST-RGP_{cd} fusion protein was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG) and was purified by affinity chromatography with a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech) as specified by the manufacturer. The $rRGP_{cd}$ was purified using the same column, following enzyme treatment with thrombin (500 NIH units) at 4°C by the procedure specified by the manufacturer.

ELISA. Serum samples obtained from immunized mice were analyzed for immunoglobulin G (IgG), IgM, and IgA antibodies against $rKGP_{cd}$ and $rRGP_{cd}$ by enzyme-linked immunosorbent assay (ELISA). Individual wells of flat-bottom 96-well plates (Nalge Nunc International, Roskilde, Denmark) were coated with 50 μl of $rKGP_{cd}$ or $rRGP_{cd}$ (10 μg/ml) in bicarbonate-carbonate buffer (pH 9.6) and incubated overnight at 4°C. The wells were blocked overnight at 4°C with

PBS containing 10% Block Ace (Dainippon Pharmaceutical Co., Osaka, Japan) and 0.05% Tween 20 (pH 7.4). Sera, in six twofold dilutions from 1:400 to 1:12,800 in PBS containing 0.05% Tween 20 (PBST), were added in triplicate to individual wells, and the mixtures were incubated for 2 h at 37°C. After the wells were washed with PBST, alkaline phosphatase-conjugated goat anti-mouse IgG, IgM, or IgA antibodies (Southern Biotechnology Associates, Birmingham, Ala.) were added and the mixtures were incubated for 2 h at 37°C. All wells were washed, and *p*-nitrophenyl phosphate in diethanolamine buffer (1 mg/ml; pH 9.4) was added. After 15 min of incubation, color development was stopped by adding 2.5 M NaOH, and the reactions were evaluated at 405 nm with a microplate reader (Titertek MK11; Flow Laboratories, McLean, Va.). The end-point titers for antigen-specific IgG, IgM, and IgA were defined as the last dilution giving an optical density at 405 nm of ≥0.1. The results are expressed as means ± standard deviations of $\log_2$ ELISA antibody titers for triplicate wells.

Isolation of specific IgG. Immune mouse sera whose end-point titers were increased to more than 6,400 were used as anti-KGP_{cd} and anti-RGP_{cd} sera. Antisera against whole cells and fimbriae of *P. gingivalis* ATCC 33277 were kindly provided by T. Fujiwara (Osaka University). Specific IgG was isolated from the antisera using a HiTrap protein G column (Amersham Pharmacia Biotech).

Immunoblot analysis. Immunized mouse serum samples were assessed for reactivity with whole-cell extracts of *P. gingivalis* ATCC 33277, W50, $rKGP_{cd}$, and $rRGP_{cd}$. Samples were separated under dissociation conditions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% polyacrylamide), and the proteins were transferred onto a nitrocellulose membrane using a Trans-Blot electrophoresis system (Bio-Rad, Hercules, Calif.). The membrane was blocked with 10% Block-Ace in PBST and washed three times with PBST. After overnight incubation with serum IgG (1:500) at 4°C, the membrane was washed three times and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (1:1,000) for 120 min at 37°C. The membrane was subsequently developed using an alkaline phosphatase substrate.

Hemoglobin-binding activity. The binding ability of the bacterial extracts to human hemoglobin was determined as described previously (20). Briefly, aliquots of the extracts (200 μl of 150 μg/ml) were immobilized on a nitrocellulose membrane (0.22 μm pore size; Bio-Dot; Bio-Rad) under mild aspiration with a Bio-Dot apparatus (Bio-Rad). The membranes were coated with 10% (vol/vol) Block Ace in PBS and incubated overnight at 4°C with specific IgG antibodies (4 μg/ml) against whole cells, KGP_{cd}, RGP_{cd}, fimbriae, and a combination of these. Nonimmunized mouse IgG and PBS were used as controls. After being washed with PBS, the samples on the membranes were incubated with human hemoglobin in PBS (0.4 mg/ml) (pH 5.5) at 4°C for 3 h. After the membranes were washed with PBS, the binding activity to hemoglobin was quantified to measure the dot intensities as described previously (20). The relative intensities were calculated based on those of the dot of bovine serum albumin BSA (30 μg) as 0% and the dots incubated without inhibitors as 100%. All assays were performed in duplicate on three separate occasions.

Proteinase activity. The inhibitory effects of anti-KGP_{cd} and anti-RGP_{cd} IgGs against Lys- and Arg-specific proteolytic activities, respectively, were investigated. The bacterial extracts (250 μg/ml) were incubated with five fivefold dilutions of IgG antibodies (100, 20, 4, 0.8, and 0.16 μg/ml) to whole cells, KGP_{cd}, or RGP_{cd} at 4°C for 2 h. Dilutions of nonimmunized mouse IgG and PBS were used as controls. After incubation, aliquots (200 μl) of the complexes were added to 790 μl of a proteinase buffer (20 mM sodium phosphate buffer containing 100 mM NaCl, 10 mM L-cysteine, and 5 mM $CaCl_2$ [pH 8.0]) and 10 μl of the synthetic substrate (10 mM stock solution) was added to a final concentration of 10 μM. Lys- and Arg-specific proteolytic activities were determined as described previously (20). The relative activity was calculated by setting those incubated with PBS as 100%.

Growth inhibition. *P. gingivalis* ATCC 33277 cells were grown in enriched TSB under iron-restricted cell conditions, and endogenous stores of iron and hemin were exhausted by two passage of a 10% inoculum into hemin-free medium. These iron-depleted cells were centrifuged and suspended with prereduced PBS. After incubation with IgGs (100 μg/ml) against whole cells, KGP_{cd}, RGP_{cd}, and fimbriae on ice under anaerobic conditions for 2 h, the cells were then inoculated into TSB supplemented with yeast extract (1 mg/ml), menadione (1 μg/ml), and 10 nM human hemoglobin as the sole iron source. Growth was monitored at 600 m in a Bausch & Lomb spectrophotometer (Shimazu Scientific Instruments, Kyoto, Japan) and documented every 6 h.

Statistical analysis. The multiple comparison was performed by Scheffe's test. The survival curves were calculated by the Kaplan-Meier method and compared by the log rank test.
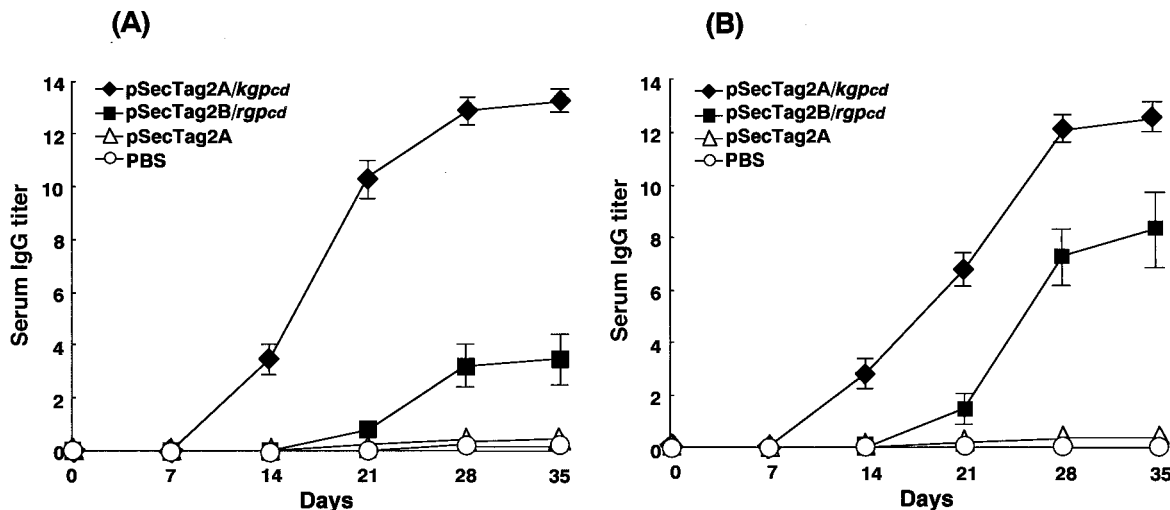
FIG. 3. Induction of IgG antibodies against rKGP$_{cd}$ and rRGP$_{cd}$ in serum from DNA-immunized mice. Mice were immunized by injection of pSecTag2A/*kgp*$_{cd}$ (50 μg) and pSecTag2B/*rgp*$_{cd}$ (50 μg), respectively. At 0, 7, 14, 21, 28, and 35 days after the primary immunization, sera were collected to evaluate specific antibody responses to rKGP$_{cd}$ (A) and rRGP$_{cd}$ (B). Control mice were immunized with intact pSecTag2A plasmid (50 μg) or PBS (50 μl). The values are expressed as means and standard deviations of log$_2$ ELISA antibody titers.

RESULTS

Immune responses to DNA vaccines. Female BALB/c mice were immunized with pSecTag2A/*kgp*$_{cd}$ or pSecTag2B/*rgp*$_{cd}$. Following the immunization, specific IgG antibodies were clearly induced, and all responses reached a plateau on day 28 (Fig. 3). Injected pSecTag2A/*kgp*$_{cd}$ elicited a significant antibody response specific to rKGP$_{cd}$ ($P < 0.0001$) (Fig. 3A), and the induced sera showed reactivities against rRGP$_{cd}$ as well ($P < 0.0001$) (Fig. 3B). Similarly, specific IgG responses were demonstrated against rRGP$_{cd}$ ($P = 0.0029$), (Fig. 3B) following immunization with pSecTag2B/*rgp*$_{cd}$, and, interestingly, a cross-reaction to rKGP$_{cd}$ was also observed (Fig. 3A). However, the titers were significantly lower than those elicited by pSecTag2A/*kgp*$_{cd}$ for reactivities with both antigens ($P < 0.0001$). Serum IgA and IgM responses were only marginal in mice immunized with either pSecTag2A/*kgp*$_{cd}$ or pSecTag2B/*rgp*$_{cd}$. No mice immunized with pSecTag2A or PBS exhibited detectable immune responses to rKGP$_{cd}$ or rRGP$_{cd}$.

Western blot analysis of antibody responses. Immunoblotting of serum antibodies elicited by the plasmid DNA immunization was performed with cellular extracts of *P. gingivalis*. Antibodies elicited by pSecTag2A/*kgp*$_{cd}$ reacted with KGP$_{cd}$ as well as RGP$_{cd}$ in whole-cell extracts of *P. gingivalis* ATCC 33277 cells (Fig. 4A, lane a). In addition, bands sized at 110, 25, and 22 kDa were found. In the *P. gingivalis* W50 preparation, a similar pattern was observed, except that a 95-kDa band was detected instead of the 110-kDa band (lane b). As shown in lanes c and d, both rRGP$_{cd}$ and rKGP$_{cd}$ were recognized by the serum. In the reaction with pSecTag2B/*rgp*$_{cd}$-immunized serum, the profiles were similar to those analyzed with pSecTag2A/*kgp*$_{cd}$-immunized serum (Fig. 4B). These results were in agreement with the results of the ELISA analysis and point to cross-reactivities between the serum IgG antibodies elicited by pSecTag2B/*rgp*$_{cd}$ and pSecTag2A/*kgp*$_{cd}$.

Inhibitory effects of the antibodies on hemoglobin binding by *P. gingivalis*. Figure 5 shows the inhibitory effects by the panel of combined IgG antibodies on hemoglobin binding ac-

tivity by *P. gingivalis*. Anti-KGP$_{cd}$, anti-whole cells, and anti-fimbria IgG antibodies significantly inhibited hemoglobin binding by the bacterial extracts ($P < 0.0005$). Furthermore, the most significant inhibition (93%) was noted in samples incubated with combinations of anti-fimbria and anti-KGP$_{cd}$ antibodies while a negligible inhibitory effect on binding was seen with anti-RGP$_{cd}$ IgG alone ($P = 0.8657$). These findings suggest that KGP$_{cd}$ and fimbriae are critical molecules for the capture of human hemoglobin by the organism.

Inhibitory effects of antibodies on the proteinase activity of *P. gingivalis*. Hydrolytic activities on the Lys-specific substrate by the cell extracts were effectively inhibited in a dose-dependent manner when the extracts were preincubated with anti-
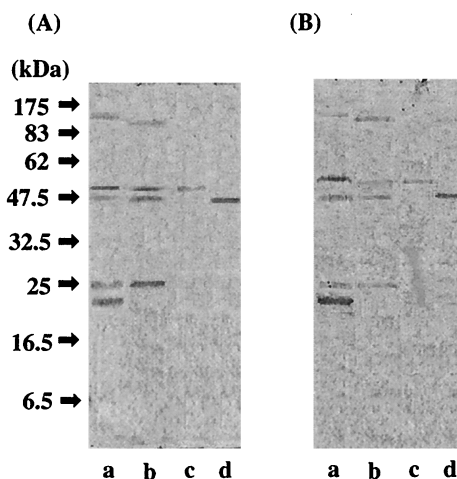

FIG. 4. Western blot analysis of serum samples. Immunoblot analyses of serum samples from mice immunized by plasmid DNA are shown. (A) Profiles probed with serum elicited by pSecTag2A/*kgp*$_{cd}$. (B) Profiles probed with serum elicited by pSecTag2B/*rgp*$_{cd}$. Lanes: a, whole-cell extracts of *P. gingivalis* ATCC 33277 (40 μg); b, whole-cell extracts of *P. gingivalis* W50 (40 μg); c, rKGP$_{cd}$ (2 μg); d, rRGP$_{cd}$ (2 μg).
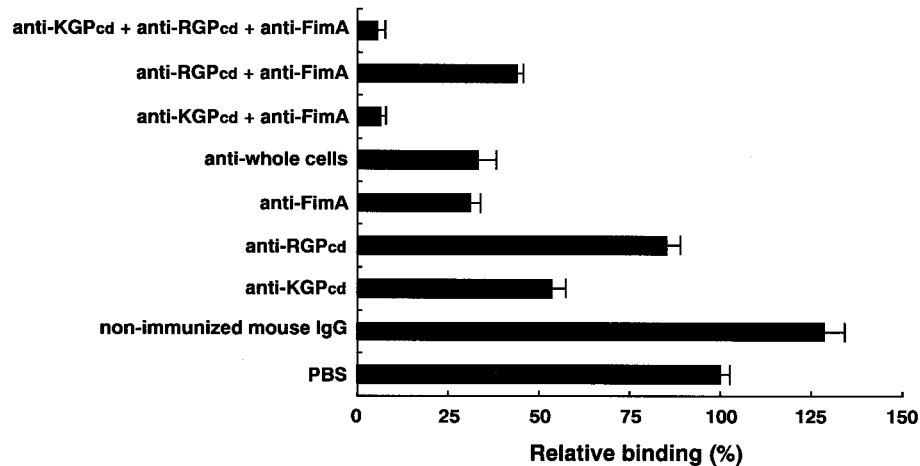
FIG. 5. Inhibitory effects of IgG antibodies on hemoglobin binding by *P. gingivalis*. Whole-cell extracts of *P. gingivalis* ATCC 33277 (30 μg), immobilized on the membrane, were incubated with IgG (4 μg/ml) against whole cells of *P. gingivalis* ATCC 33277, rKGP$_{cd}$, rRGP$_{cd}$, fimbriae, and combinations of these and then incubated with human hemoglobin (0.4 mg/ml) at 4°C for 3 h. The densitometric values are expressed as percentages of those obtained with PBS (100%). The experiments were performed twice in triplicate, and the values are expressed as means and standard deviations.

KGP$_{cd}$ or anti-RGP$_{cd}$ IgG antibody dilutions ($P < 0.0001$ and $P < 0.0001$, respectively) (Fig. 6A). There was no significant difference between the inhibitory effects displayed by anti-KGP$_{cd}$ and anti-RGP$_{cd}$ antibodies. On the Arg-specific substrate, aminolytic activities were unaffected by a 4-μg/ml concentration of anti-KGP$_{cd}$ or anti-RGP$_{cd}$ IgG antibodies but were significantly inhibited by concentrations over 20 μg/ml ($P = 0.0126$ and $P = 0.0026$, respectively) (Fig. 6B). The two antibodies showed similar inhibitory effects. However, there was no apparent trend in the inhibition by anti-whole cells or nonimmunized IgG (Fig. 6).

Growth delay by the antibodies. The growth of iron-depleted *P. gingivalis* cells was evaluated in basal medium supplemented with human hemoglobin as the only iron source, following preincubation with anti-KGP$_{cd}$ and/or anti-RGP$_{cd}$ IgG antibodies. Control cells treated with PBS reached a plateau after 82 h of incubation, while preincubation with anti-KGP$_{cd}$ or anti-RGP$_{cd}$ significantly delayed the growth at 82 h ($P = 0.0375$ and $P = 0.0269$, respectively) and caused cell growth to reach the plateau at 110 h of incubation (Fig. 7). In contrast, anti-whole cells or nonimmunized IgG showed no marked effect on the cell growth.

Effects of plasmid DNA immunization on lethal challenge. Female BALB/c mice immunized with pSecTag2A/*kgp*$_{cd}$ or pSecTag2B/*rgp*$_{cd}$ were intraperitoneally challenged with viable *P. gingivalis* cells. The immunization of plasmid DNA con-
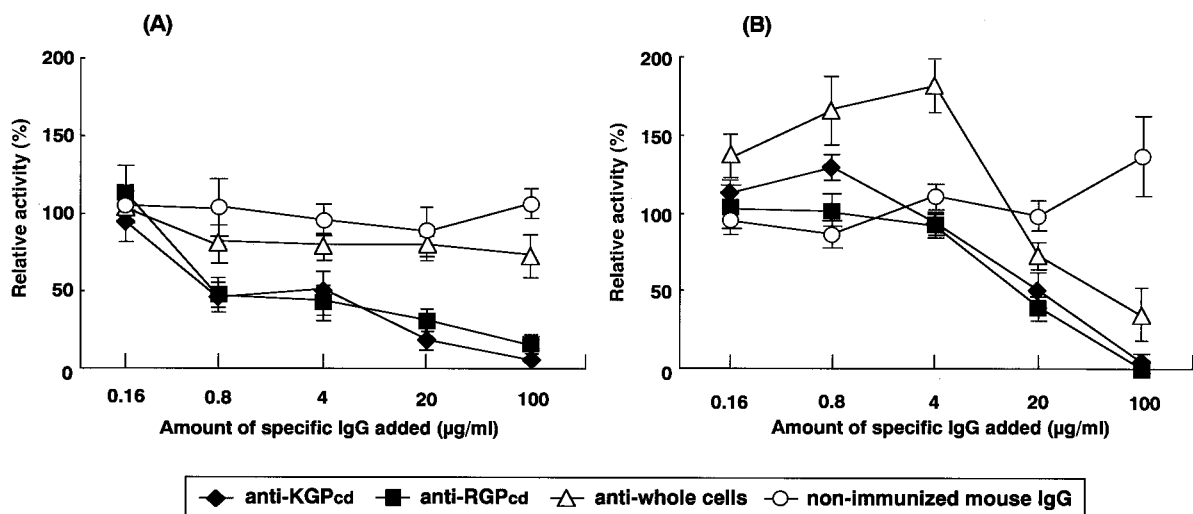

FIG. 6. Inhibitory effects of the IgG antibodies on proteinase activity of *P. gingivalis*. Whole-cell extracts of *P. gingivalis* ATCC 33277 (0.25 μg/μl) were serially incubated with fivefold dilutions of IgG antibodies (×1; 100 μg/ml, ×1/5; 20 μg/ml, ×1/25; 4 μg/ml, ×1/125; 0.8 μg/ml, ×1/625; 0.16 μg/ml) at 4°C for 2 h. The nonimmunized mouse IgG dilution and PBS were used as controls. The Lys-specific (A) and Arg-specific (B) cysteine proteinase activities of the complexes were assayed with Boc-Val-Leu-Lys-MCA and Boc-Gln-Ala-Arg-MCA, respectively. Relative activities were calculated by setting those incubated with PBS as 100%. All assays were performed in triplicate on three separate occasions, and the values are expressed as means and standard deviations.
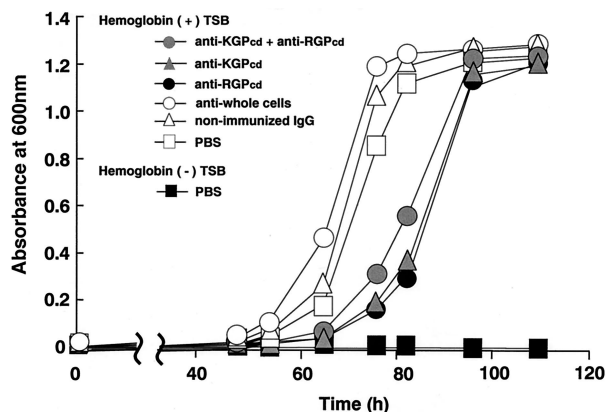
FIG. 7. Effects of IgG antibodies on the growth of *P. gingivalis* in medium supplemented with hemoglobin as the sole iron source. Iron-depleted cells of *P. gingivalis* ATCC 33277 were incubated with various IgG antibodies (100 µg/ml) on ice under anaerobic conditions for 2 h and then inoculated into medium supplemented with human hemoglobin as the sole iron source. Control cells were preincubated with PBS and then inoculated into TSB with or without hemoglobin. All assays were performed in triplicate on three separate occasions.
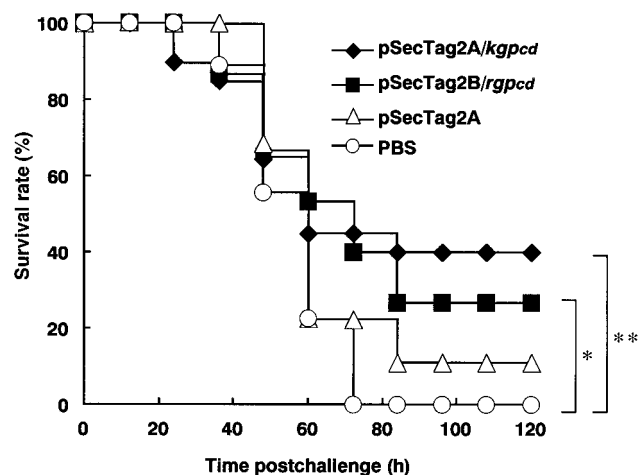

FIG. 8. Effects of plasmid DNA immunization against lethal challenge with *P. gingivalis* in mice. Female BALB/c mice immunized with plasmid DNA were intraperitoneally challenged with viable *P. gingivalis* W50 cells (9×10^9 CFU). Intact pSecTag2A and PBS were used as controls. The log rank test was used for calculation of *p* values. *, $P = 0.0069$; **, $P = 0.0088$.

ferred a significant amount of protection against the lethal challenge compared with that in control animals given PBS. All mice in the control group died within 72 h, while 43% in the pSecTag2A/kgp_{cd}-immunized group and 27% in the pSecTag2B/rgp_{cd}-immunized group survived until the end of the experiment (120 h) ($P = 0.0088$ and $P = 0.0069$, respectively) (Fig. 8). Although the degree of inflammatory features varied with the individual mice, the immunization clearly lessened such infectious symptoms as eroded skin lesions on the abdomens, as well as severe cachexia with ruffled hair and hunched bodies (Table 1).

DISCUSSION

The major proteinase, KGP and RGP are believed to play a critical role in the pathogenesis of *P. gingivalis* (1, 12, 17, 21, 22, 28, 42, 43). Both are produced as polyprotein moieties and are composed of several functional domains. KGP polyprotein is formed from KGP_{cd} and the hemagglutinin/adhesin domain (HGP27/44, HGP15, HGP17, and HGP27) (31, 33). In our previous study, it was shown that KGP_{cd} binds to human hemoglobin with a significant affinity, which is mediated through an active region(s) distinct from those for proteinase activity. Other studies showed that HGP15 and fimbriae are also capable of binding to hemoglobin (2, 27). Here we attempted to investigate the exact role of KGP_{cd} in hemoglobin binding by *P. gingivalis* using plasmid DNA-elicited antibodies.

The antibodies elicited by pSecTag2A/kgp_{cd} clearly reacted with KGP_{cd}, RGP_{cd}, and their polyproteins on immunoblotting. Although unknown bands of 25 and 21 kDa were probed by the antibodies, as shown in a previous study using anti-RGP peptide (34), HGP15 was not detected. The KGP_{cd} IgG significantly inhibited the hemoglobin-binding abilities of *P. gingivalis*. Further, the combination of anti-fimbria and anti-KGP_{cd} antibodies almost completely inhibited hemoglobin binding by the organism. The kgp_{cd}-deficient mutants reportedly increased the expression of fimbriae compared to that caused by the parent strain, but the mutants failed to form black-pig-

mented colonies (6). These results strongly suggest that the hemoglobin binding for hemin uptake of *P. gingivalis* is mediated by KGP_{cd}. Fimbriae could have no association with hemin storage but might act as a carrier to pass hemoglobin to KGP_{cd}.

Recent studies by other laboratories support our findings. In the spontaneous mutants of *P. gingivalis* without pigmentation, the $5'$ end of the kgp_{cd} gene was found to be deleted whereas the portion encoding the KGP hemagglutinin domain was innately present (7). These spontaneous kgp_{cd} mutants have markedly decreased binding ability to hemoglobin. It was also shown that inactivation of kgp_{cd} with an IS element generated these mutants, forming a white colony on blood plates as a result of a hemin-hemoglobin defect (6, 23, 40). Chen et al., when speculating that HGP15 is a hemoglobin-binding protein, suggested that proteolytic activity of KGP_{cd} is essential to cleave HGP15 from the its polyproteins and thus the kgp_{cd} mutants are unable to produce HGP15 protein, resulting in the white colonies (6). However, RGP_{cd} reportedly can be used instead of KGP_{cd} to cleave HGP15 and support the expression of its activity (30, 37). Furthermore, it was reported that kgp_{cd}-deficient mutants produced HGP15 slowly after 7 days of incubation but mutants with the mature HGP15 formed white or

TABLE 1. Disease status of *P. gingivalis* infection in immunized mice

Mouse group	Total no. of mice	No. of mice with cachexia[a]/total no.				No. of mice with eroded skin lesion[b]/total no.	
		None	Slight	Moderate	Severe	0–4.0 cm²	>4.0 cm²
pSecTag2A/kgp_{cd}	21	0/21	6/21	6/21	9/21	16/21	5/21
pSecTag2B/rgp_{cd}	19	0/19	2/19	5/19	12/19	14/19	5/19
pSeqTag2A	9	0/9	0/9	0/9	9/9	6/9	3/9
PBS	10	0/10	0/10	0/10	10/10	1/10	9/10

[a] Cachexia was defined as ruffled hair, hunched bodies, and weight loss.
[b] Lesion size was determined and expressed as the average maximum diameter achieved during a 14-day postinfection period.

beige colonies (30). It was also demonstrated that kgp_{cd} mutants exhibited HGP15 on the cell surface after incubation in enriched brain heart infusion broth for 48 h (37). The nonpigmentation of the various mutants seems to be consistent with the absence of KGP_{cd}, regardless of the presence of HGP15. These results strongly suggest that KGP_{cd} acts as a critical receptor in hemin-hemoglobin uptake by *P. gingivalis* than HGP15. It might be hypothesized that the hemin-hemoglobin uptake by *P. gingivalis* is mediated by the following pathway: fimbriae and KGP_{cd} bind to hemoglobin, then KGP_{cd} holds the captured hemoglobin for denaturing, and HGP15 and HumR (39) act in the subsequent events such as storage of released hemin. Further study is necessary to understand these details.

The antibodies generated by pSecTag2A/kgp_{cd} and pSecTag2B/rgp_{cd} recognize recombinant and native proteins of KGPcd and RGPcd, respectively. Moreover, both antibodies were found to markedly inhibit Lys-specific and Arg-specific cysteine proteinase activities of *P. gingivalis*, without significant inefficiencies. These cross-reactivities between the two gingipains were also observed in another study (29). Recently, Curtis et al. analyzed the homology between KGP and RGP by using the diagon plot comparison with a window of 30 amino acids and a stringency of 11 (9). This comparison of the deduced amino acid sequence of the Lys-specific proteinase with that of Arg-specific proteinase reveals considerable conservation in both the propeptide and catalytic domains, suggesting a common ancestral gene for these two loci. The catalytic domains of the two gingipains have only a limited identity (27%): however, both polypeptides have a high similarity of sequences corresponding to a region encompassing the catalytic cysteine residues (10 aa), as well as in the carboxy-terminal region (30 aa) (11, 33). These similar motifs result in cross-reactivities among the antibodies present. However, hemoglobin binding is not influenced by the cross-reactivities among the antibodies; therefore, this interaction would be mediated by a motif distinct from the shared region for proteinase activity. It was reported that immunization of the whole RGPcd molecule failed to elicit a marked immune response (13, 29). This finding could explain why pSecTag2A/kgp_{cd} elicited a stronger response than pSecTag2B/rgp_{cd} as shown in Fig. 3.

Anti-KGPcd IgG was expected to delay the growth of *P. gingivalis* due to its inhibitory effect on hemin uptake when the cells were grown with human hemoglobin as the only iron source. Therefore, *P. gingivalis* was grown following preincubation with anti-KGP_{cd} and/or anti-RGP_{cd} IgGs. Cell growth was significantly delayed by anti-KGP_{cd} as anticipated. However, contrary to our expectation, anti-RGP_{cd} also revealed a remarkable effect on cell growth. This may be explained by previous findings that RGP is involved mainly in the proteolysis of nutritive substances for growth (21, 37). The delay could have first been due to the inhibition of hemoglobin uptake by anti-KGP_{cd}, while the prevention of proteolytic abilities by antibodies against KGP_{cd} or RGP_{cd} would also have supported the delay, because of their ability to inhibit the generation of small nutritive peptides by *P. gingivalis*.

In our preliminary experiments, mice did not die but developed a localized abscess on their abdomen when infected with 10^{10} CFU of *P. gingivalis* ATCC 33277, whereas they all died when infected with the same inoculum of strain W50. With respect to the amino acid sequences and functions of Lys- and

Arg-specific proteinases, strains ATCC 33277 and W50 were found to be very similar to each other (9). Within the catalytic domain, these strains have a highly conserved identity: the homologies of the amino acid sequences are 94.7% between Lys-specific proteinases (KGP of ATCC 33277 and PrtK of W50), 91.6% between Arg-specific proteinases (RGP and PrtRI), and 98.0% between another kind of Arg-specific proteinases (RgpB and PrtRII). Therefore, we used strain W50 for the challenge experiments. The immunization with pSecTag2A/kgp_{cd} and pSecTag2B/rgp_{cd} showed marked effects on clinical features and survival rates compared to the controls. Immunization with both plasmid DNAs is likely to inhibit the proteolytic abilities of *P. gingivalis* in vivo. In addition, the inhibitory effects on bacterial hemin uptake may result in effective protection by pSecTag2A/kgp_{cd} immunization. This may explain why pSecTag2A/kgp_{cd} was more effective than pSecTag2B/rgp_{cd}. The intact pSecTag2A without kgp_{cd} or rgp_{cd} also decreased the lesion size, which may be attributable to CpG sequences contained in the plasmid (35). The CpG motifs are known to possess T-helper type 1 immunostimulatory activity and to stimulate monocytes and macrophages to produce a variety of cytokines including interleukin-12, tumor necrosis factor alpha, and alpha/beta interferon. The CpG motifs can also stimulate the production of interleukin-6, which, in turn, promotes B-cell activation and IgM secretion. These immunostimulatory activities of the CpG motifs could have suppressed the lesion formation. Several efforts have been previously made to generate effective vaccines targeting the RGP_{cd} molecule. However, most of those studies suggested that the whole RGP_{cd} molecule was not a suitable immunogen to elicit a marked immune response (13, 29). These findings may also explain the weaker effects of immunization with pSecTag2B/rgp_{cd}. As a result, it is suggested that KGP_{cd} is a more promising candidate for future vaccination than RGP_{cd}.

In summary, the present study found that KGP_{cd} acts as a hemoglobin-binding protein of *P. gingivalis* and suggests that it may also be a possible immunogen to induce a protective response to *P. gingivalis* infection.

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