INFECTION AND IMMUNITY, May 2001, p. 2858–2864 Vol. 69, No. 5
0019-9567/01/$04.00+0 DOI: 10.1128/IAI.69.5.2858–2864.2001
Copyright © 2001, American Society for Microbiology. All Rights Reserved.

Arg-Gingipain A DNA Vaccine Induces Protective Immunity against Infection by *Porphyromonas gingivalis* in a Murine Model

HIDEO YONEZAWA,* KAZUYUKI ISHIHARA, AND KATSUJI OKUDA

Oral Health Science Center, Department of Microbiology, Tokyo Dental College, Mihama-ku, Chiba-City, Chiba 261-8502, Japan

Received 24 October 2000/Returned for modification 10 January 2001/Accepted 5 February 2001

Arginine-specific cysteine proteinases (RgpA and RgpB) produced by the periodontal pathogen *Porphyromonas gingivalis* are suspected virulence factors and are involved in interrupting host defense mechanisms as well as in penetrating and destroying periodontal connective tissues. To induce a protective immune response against *P. gingivalis*, we constructed an *rgpA* DNA vaccine. BALB/c mice were immunized intradermally by Gene Gun with plasmid DNA carrying *rgpA*. Antibody responses against *P. gingivalis* were determined by an enzyme-linked immunosorbent assay. The *rgpA* DNA vaccine induced high levels of serum antibodies against *P. gingivalis*. Sera from the *rgpA* DNA vaccine-immunized mice diminished the proteolytic activity of RgpA and RgpB and inhibited the binding of *P. gingivalis* to a type I collagen sponge. Moreover, the sera effectively reduced the hemagglutination of *P. gingivalis*, indicating that the hemagglutinin activity of the organism is associated with RgpA. We found with a murine abscess model that mice immunized with the *rgpA* DNA vaccine were resistant to an invasive *P. gingivalis* W50 challenge. These results suggest that the *rgpA* DNA vaccine induced specific antibodies against the enzyme and that this vaccine could confer protective immunity against *P. gingivalis* infection.

Porphyromonas gingivalis, a gram-negative anaerobic bacterium, has been isolated frequently from subgingival lesions in patients with adult periodontitis, and the microorganism is thought to be a major etiologic bacterium associated with the progression of human periodontal disease (33). A number of virulence factors have been implicated in the pathogenicity of *P. gingivalis* (20), and particular emphasis has placed focused on the cysteine proteinases (29). Cysteine proteinases have been isolated from culture supernatants, vesicle membrane fractions, and cell extracts of *P. gingivalis* (10, 26, 34). A recent report indicated that most of this activity is derived from either arginine- or lysine-specific cysteine proteinases (30). These enzymes may participate in pathogenicity by activating host proenzymes such as plasminogen or by exposing host cell cryptotopes, as well as by altering blood clotting (4, 14, 38). In addition, arginine-specific cysteine proteinases (Arg-gingipains) can degrade a variety of host proteins such as connective tissue components (36). The host immune response in these regions may be further dysregulated by the proteinases, since they can degrade immunoglobulins (9).

Arg-gingipain-encoding genes have been cloned from various *P. gingivalis* strains (23, 27), and sequence analysis has revealed that two separate Rgp-encoding genes (*rgpA* and *rgpB*) are located on the chromosome of *P. gingivalis*. The RgpA enzyme consists of a propeptide domain, a catalytic domain, and a hemagglutinin domain, while RgpB consists of a propeptide domain and a catalytic domain. It is important to note that the catalytic domains of the two genes are almost identical (3). Components of the hemagglutinin domain were designated HGP 44, HGP 15, HGP 17, and HGP 27 (23, 27). Most of the C-terminal hemagglutinin domain is absent in RgpB. A recent report (21) has shown that mutations causing defects in the *rgpA* and/or *rgpB* gene affect the growth of *P. gingivalis* and its ability to bind to epithelial cells and gram-positive bacteria, as well as extracellular matrix proteins. These defects appeared to be related to expression of cell surface structures such as fimbriae and vesicles (21, 37). Moreover, these mutants showed decreased hemagglutinating activities.

Trials for induction of protective immune responses against *P. gingivalis* have been performed using pathogenic factors such as fimbriae, Arg-gingipain, and hemagglutinin B (12, 16, 17). A recent report (35) indicated that noninvasive *P. gingivalis* strains such as ATCC 33277 and 381 are fimbriated, whereas invasive strains such as W50 are reported to be sparsely fimbriated, with shorter fimbriae that are devoid of hemagglutinating activity. Virulence factors common to both invasive and noninvasive strains of *P. gingivalis* are the Arg- and Lys-gingipains. A vaccine targeting these proteinases may therefore provide protection against both invasive and noninvasive strains of *P. gingivalis*. In a mouse model, immunization with a multiple-antigen peptide corresponding to the catalytic domain of Rgp induced the production of antibodies which afforded mice protection from *P. gingivalis* infection (12). Interestingly, immunization with a peptide corresponding to the active site or with several peptides corresponding to the hemagglutinin domain of the proteinase conjugated to diphtheria toxoid also attenuated lesion formation in a mouse model (22).

The ability to induce an immune response to a protein antigen by administration of plasmid DNA encoding the antigen has been successfully demonstrated in animal models (5). DNA vaccines consist of a eukaryotic expression vector containing a target gene of interest (6, 40). A mammalian promoter drives gene expression, and transcription is terminated by a mammalian polyadenylation signal in mammalian cells.

* Corresponding author. Mailing address: Department of Microbiology, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba-City, Chiba 261-8502, Japan. Phone: 81-43-270-3742. Fax: 81-43-270-3744. E-mail: yonezawa@tdc.ac.jp.

TABLE 1. Oligonucleotide sequences[a] of PCR primers

Primer	Sequence	Amplified fragment
RgpA1	5′-GGGGGGCTAGCGACCATGAAAAACTTGAACAAGTTTGTTTCGATTGC-3′	949–6082
RgpA2	5′-GGGGGCTCGAGCTCCGAGTCCAAGACAGAATTAC-3′	
RgpART1	5′-GATTGCTCTTTGCTCTTCCTTA-3′	975–1688
RgpART2	5′-TACTTTTTGGCTACGATGACGA-3′	

[a] Restriction site sequences are underlined.

While DNA vaccination with a single bacterial gene is ostensibly still a subunit approach to vaccination, it is particularly attractive compared to administration of a preformed protein antigen because the immunogen of interest is actively synthesized in vivo in transfected cells. The immunogen is available for expression by major histocompatibility complex class I and class II molecules, thereby inducing specific cellular (both $CD4^+$ and $CD8^+$ T-lymphocyte) and antibody responses in immunized hosts (18, 39).

In the study reported here, we constructed a DNA vaccine to express RgpA directly in mice by a Gene Gun-mediated DNA vaccine. In addition, we examined the ability of this vaccine to induce a protective immune response against *P. gingivalis* in a mouse model.

MATERIALS AND METHODS

Bacterial strains. *P. gingivalis* strains ATCC 33277, W83, and W50 were grown in Trypticase soy broth (Beckton Dickinson Microbiology Systems, Cockeysville, Md.) supplemented with 5 μg of hemin (Sigma Chemical Co., St. Louis, Mo.) per ml and 0.5 μg of menadione (Wako Pure Chemical Industries, Osaka, Japan) per ml under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2). *Escherichia coli* XL1-Blue (Stratagene, La Jolla, Calif.) and BL21 (Gibco BRL, Gaithersburg, Md.) were grown in Luria-Bertani (LB) broth or on LB agar plates.

Construction of a DNA vaccine. The region encoding the catalytic domain and hemagglutinin domain of Arg-gingipain A was amplified by PCR for *P. gingivalis* ATCC 33277 genomic DNA. PCR primers based on the *rgpA* gene sequence with restriction site sequences are shown in Table 1; forward primer (RgpA1) incorporated an *Nhe*I site, whereas the reverse primer (RgpA2) incorporated an *Xho*I site. PCR mixtures contained 1 μl of genomic DNA, 5 μl of 10× PCR buffer (Takara Shuzo, Tokyo, Japan), 200 mM $MgCl_2$, and 1.25 U of Z-*Taq* (Takara Shuzo). PCR amplification for the microorganisms was performed using a GeneAmp 9600 thermal cycler (Perkin-Elmer, Foster City, Calif.). The PCR temperature profile included denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 98°C for 5 s, annealing at 55°C for 10 s, and extension at 72°C for 80 s, followed by 7 min of heating at 72°C. The 5,133-bp amplified *rgpA* gene was digested with *Nhe*I and *Xho*I and ligated with vector pVAX1 (Invitrogen, Carlsbad, Calif.). The plasmid obtained (see Fig. 1) was transformed into *E. coli* XL1-Blue by electroporation, and the transformants were selected on LB agar plates containing 60 μg of kanamycin per ml.

Mouse vaccination. Six-week-old female BALB/c mice (Sankyo Laboratories Service Co. Inc., Tokyo, Japan) were used in this study. Approval to conduct these studies was obtained from the Animal Use Committee of Tokyo Dental College (Chiba, Japan). Gold particles were coated with the *rgpA* DNA vaccine according to the manufacturer's instructions. Briefly, 2.5 μg of plasmid DNA was precipitated onto 500 μg of 1.6-μm-diameter gold beads in 1.0 M $CaCl_2$ and 100 mM spermidine. For each vaccination, mice received a total of 2.5 μg of DNA administered with a Helios Gene Gun at a helium gas pressure of 400 lb/in^2 on the skin of the abdomen (15). Mice were immunized at weeks 0, 1, 2, 3, and 4. Blood samples were obtained from mice on days 0 (before the first vaccination), 7 (before the second vaccination), 14 (before the third vaccination), 21 (before the fourth vaccination), 28 (before the fifth vaccination), 35, and 42.

For detection of RgpA expression in immunized mice, mouse abdomen skin was isolated under anesthesia at 5 days after the first immunization. The RNA was isolated by an RNeasy RNA isolation kit (Qiagen Inc., Valencia, Calif.), and reverse transcription-PCR (RT-PCR) amplification was done using a One Step RNA PCR kit (Takara Shuzo) according to the supplier's instructions. Primers specific for the *rgpA* gene sequences are shown in Table 1 (RgpART1, RgpART2). The RT-PCR temperature profile included, first, RT at 50°C for 30 min, then denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 60 s, and finally, a 7-min step at 72°C. The PCR products were electrophoresed on a 1% agarose gel containing 0.2 mg of ethidium bromide per ml for staining.

ELISA. Anti-RgpA immunoglobulins in serum were detected by an enzyme-linked immunosorbent assay (ELISA) technique. Briefly, 20 μg of sonic extracts of *P. gingivalis* per well in 50 μl of 50 mM carbonate buffer (pH 9.6) was used to coat each well of a 96-well plate at 4°C overnight. To prepare sonic extracts, washed cells were disrupted following sonication (Sonifier 250; Branson Sonic Power Company, Danbury, Conn.). Wells were blocked with 200 μl of 3% bovine serum albumin in phosphate-buffered saline (PBS) at 37°C for 60 min. Duplicate serial twofold dilutions of samples in an appropriate range for the particular analysis were applied to the wells and incubated at 37°C for 60 min. The secondary antibody, peroxidase-labeled goat anti-mouse immunoglobulin (Dako A/S, Glostrup, Denmark), was used at a 1:2,000 dilution. After development, absorbance at 490 nm was measured with a microplate reader (Bio-Rad Laboratories, Hercules, Calif.). The endpoint titers for antigen-specific immunoglobulins were defined as the last dilutions giving an optical density at 490 nm of ≥0.2.

Western blot assay. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses were performed on 10 to 20% gradient Micro Slab gels (Daiichi Pure Chemical Co., Tokyo, Japan) with the Laemmli buffer system (19). For Western blot assays, sonic extracts of *P. gingivalis* ATCC 33277 and recombinant RgpA proteins were utilized as samples. The domains of RgpA had been expressed from *E. coli* BL 21 with a pET system according to the supplier's instructions (Novagen, Inc., Madison, Wis.). Three plasmids, i.e., pET32 (containing an inserted DNA sequence for the catalytic domain corresponding to amino acid residues 228 to 719), HGP 44 (corresponding to amino acid residues 720 to 1136), and the HGP complex (HGP 15, 17, and 27) (corresponding to amino acid residues 1137 to 1704), were kindly provided by S. Inagaki (Tokyo Dental College). The molecular masses of recombinant products of the catalytic domain, HGP 44, and the HGP complex are approximately 17 kDa greater than originally because these domains were fused with histidine tags and thioredoxin in the expression vectors. The sonic extracts were loaded onto SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, Mass.) using Trans-Blot (Bio-Rad Laboratories). After transformation, the polyvinylidene difluoride membranes were blocked with 3% bovine serum albumin in PBS at 37°C for 60 min and the membranes were incubated in diluted sera (1:500) from the *rgpA* DNA vaccine-immunized mice at 37°C for 60 min. Nonimmunized mouse serum was also used, as a negative control. After washing of membranes, peroxidase-labeled goat anti-mouse immunoglobulins (Dako A/S) were used at a 1:2,000 dilution as secondary antibodies. The blots were developed after washing.

Enzyme activity assay. Proteolytic enzyme activity was measured using the synthetic substrate *N*-α-benzoyl-L-arginine-*p*-nitroanilide (BA*p*NA) (Sigma Chemical Co.) (26). Sonic extracts of *P. gingivalis* were pretreated with mouse serum samples at 1/10, 1/20, and 1/50 dilutions at 37°C for 30 min. An aliquot of 20 μl of pretreated samples was added to 150 μl of the reaction mixture, which consisted of 100 mM Tris-HCl buffer (pH 7.5) containing 5 mM dithiothreitol and 1.0 mM BA*p*NA. The mixture was incubated at 37°C for 15 min, and then the reaction was stopped by adding 50 μl of 20% acetic acid. Release of *p*-nitroaniline was determined by its absorbance at 405 nm.

Assay of binding to type I collagen. For labeling, *P. gingivalis* ATCC 33277 or W83 was incubated with 5 μCi of [^{3}H]thymidine (NEN LIFE Science Products, Boston, Mass.) per ml at 37°C for 24 h anaerobically. Binding inhibition assays of immunized mouse serum were performed using honeycomb type I collagen sponges (Koken, Tokyo, Japan). Labeled bacterial cells (10^8 cells per ml) in 200 μl of buffered KCl were treated with sera immunized with the *rgpA* DNA vaccine or with nonimmunized sera at a 1:100 dilution at room temperature for 1 h. Then the pretreated cells were added to 48-well plates containing honeycomb type I

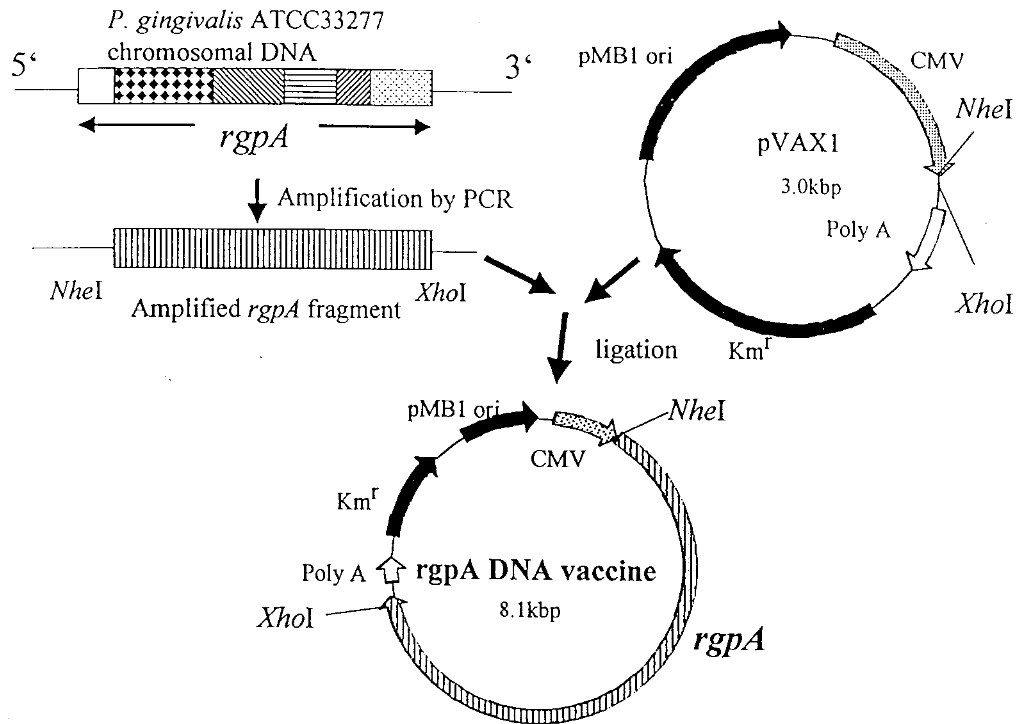

FIG. 1. Construction of *rgpA* DNA vaccine. A 5.1-kb amplified *rgpA* gene fragment was cloned into the *NheI* and *XhoI* sites of plasmid pVAXI. CMV, cytomegalovirus promoter; Poly A, polyadenylation signal; □ ▦ ▧ ▤ ▨ ▩, regions of the proprotein, catalytic domain, and HGP 44, HGP 15, HGP 17, and HGP 27, components of the hemagglutinin domain, respectively.

collagen sponges. The mixtures were incubated using the rotator at room temperature for 1 h. The collagen sponges were washed three times with buffered KCl and then transferred to a vial, the radioactivity of each sponge was determined by direct scintillation counting, and the percent adsorption for each was calculated.

Hemagglutination assay. Overnight cultures of *P. gingivalis* ATCC 33277 were centrifuged, washed three times with PBS, and resuspended in PBS. Serum samples from mice immunized with the *rgpA* DNA vaccine or from nonimmunized mice were diluted in a twofold series with PBS. Aliquots of 25 μl of each dilution were mixed with equal volumes of the bacterial suspension. These mixtures were applied to a round-bottomed microplate (Iwaki, Chiba, Japan) and incubated at room temperature for 30 min. After incubation, each mixture was added to a 25-μl volume of sheep erythrocyte suspension (2% in PBS) and incubated at room temperature for 180 min. The hemagglutination titer was determined as the last dilution exhibiting full agglutination.

Murine abscess model. In order to examine the protective effect of immunization with the *rgpA* DNA vaccine against an invasive *P. gingivalis* W50 challenge, a murine lesion model like the one described by Ebersole et al. (8) was utilized. Overnight cultures of *P. gingivalis* W50 were harvested, washed three times with PBS, and resuspended in PBS. After the 48th day of the first immunization, 10 mice immunized with the *rgpA* DNA vaccine were challenged with 10^9 cells of *P. gingivalis* W50 subcutaneously in the abdomen and the lesion sizes were measured for 5 days. Nonimmunized mice were also challenged, as a control.

RESULTS

Construction of the *rgpA* DNA vaccine and expression of RgpA. Figure 1 shows the construction of an *rgpA* DNA vaccine. The *rgpA*-carrying DNA fragments obtained by PCR were digested with each restriction endonuclease and ligated into *Nhe*I- and *Xho*I-digested plasmid pVAX1. The resultant plasmid, i.e., the *rgpA* DNA vaccine, was confirmed by PCR and DNA sequencing (date not shown).

For evaluating expression of the *rgpA* gene in mice, BALB/c mice were immunized by Gene Gun injection of the *rgpA* DNA vaccine on the skin of the abdomens and the injected areas were isolated 5 days after the first immunization. After isolation of RNA from the tissue, expression of the *rgpA*-specific mRNA around the immunized regions was analyzed by RT-PCR (Fig. 2). The *rgpA*-specific mRNA of 713 bp was detected in the muscle tissue of the mice.

Response of immunoglobulins to *P. gingivalis*. BALB/c mice were immunized by Gene Gun injection of the *rgpA* DNA vaccine into the abdomen and given booster injections four times at 7, 14, 21, and 28 days after the primary inoculation. The dose given was 2.5 μg of DNA per shot. Serum samples were taken at 42-day periods, and total anti-RgpA immunoglobulins were measured by ELISA (Fig. 3). *P. gingivalis*-specific antibodies were detected 14 days after the primary vaccination, with an increase in titer following each booster shot and an increase thereafter. This analysis showed that sera from mice immunized with the *rgpA* DNA vaccine had elevated levels of antibody against *P. gingivalis*.

Western blot analysis. To characterize the immune responses against RgpA, sera from mice were analyzed by Western blotting (Fig. 4A). At 2 weeks after the final Gene Gun injection, sera from mice immunized with the *rgpA* DNA vaccine reacted with sonic extracts of *P. gingivalis*. Interestingly, these sera had strongly immunoreactive responses to the 45-, 44-, 27-, 17-, and 15-kDa protein bands. These protein bands correspond to the sizes of the catalytic domain and the HGP 44, HGP 27, HGP 17, and HGP 15 components of the hemagglutinin domain, respectively. In addition, the sera reacted

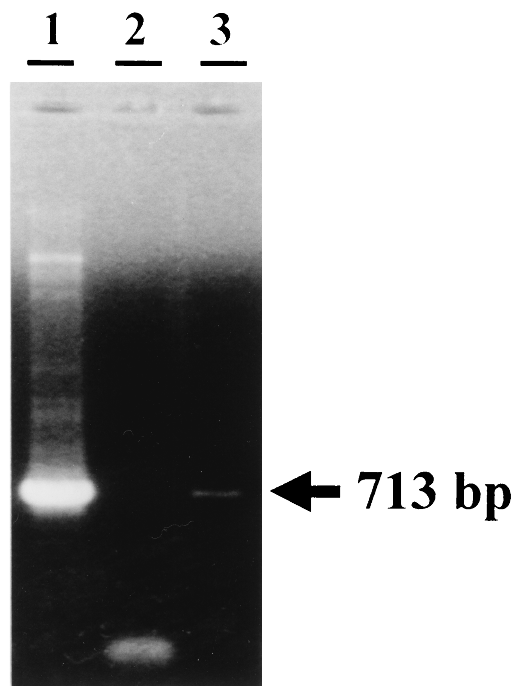

FIG. 2. Expression of *rgpA*-specific mRNA around the immunized regions of mice. RNA was purified from transfected cells of mice and amplified by RT-PCR using *rgpA*-specific primers. Lanes: 1, plasmid pVAX1 containing *rgpA* gene (positive control); 2, *rgpA* DNA vaccine plasmid-transfected cells without RT reaction (negative control); 3, *rgpA* DNA vaccine plasmid-transfected cells.

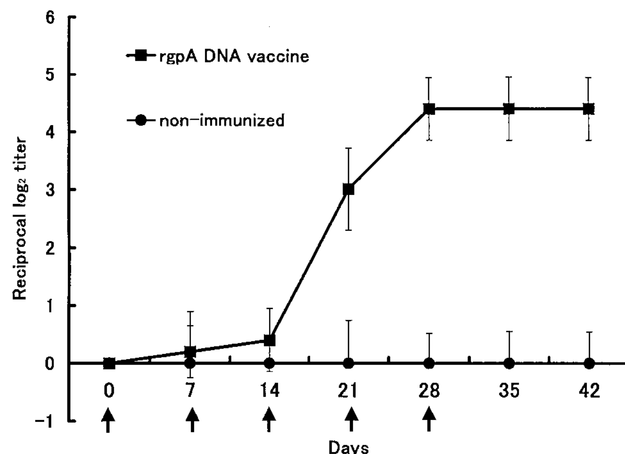

FIG. 3. Induction of *P. gingivalis*-specific antibodies in sera from *rgpA* DNA vaccine-immunized mice. Serial dilutions were used to measure the endpoint titers ($\geq$0.2). The *rgpA* DNA vaccine was injected, as shown by arrows, in a total of five inoculations. The results are means $\pm$ standard deviations of $\log_2$ ELISA antibody titers. The variation in the endpoint titers was within 1 twofold serial dilution.

with 62-, 61-, and 76-kDa recombinant proteins corresponding to the catalytic domain, HGP 44, and the HGP complex of RgpA, respectively (Fig. 4B). These results indicate that immunization with the *rgpA* DNA vaccine using a Gene Gun induced specific antibodies to all domains of RgpA.

Inhibition of enzyme activity of *P. gingivalis*. To investigate whether the anti-RgpA antibodies induced by the DNA vaccine inhibit the proteinase activity of *P. gingivalis*, the BA*p*NA test was carried out. Sera from immunized mice exhibited a greater ability to inhibit the proteinase activity than those from nonimmunized mice. The *rgpA* DNA vaccine-immunized mouse serum samples diluted at 1/10, 1/20, and 1/50 inhibited proteinase activity by 25.0, 28.8, and 32.6%, respectively, compared with nonimmunized serum samples.

Inhibition of *P. gingivalis* binding. Effects of the sera from mice immunized with the *rgpA* DNA vaccine on the binding of *P. gingivalis* fimbriated strain ATCC 33277 or nonfimbriated strain W83 to type I collagen were evaluated using honeycomb type I collagen sponges. All tested sera inhibited the binding of *P. gingivalis* whole cells to the collagen (Table 2). However, inhibition by sera from mice immunized with the *rgpA* DNA vaccine was statistically greater than that by sera from nonimmunized mice.

Inhibition of hemagglutinating ability of *P. gingivalis*. One of the significant features of *P. gingivalis* is its ability to agglutinate erythrocytes. Hemagglutinating activity is one of the functions of the Rgp protein. To determine whether the *rgpA* DNA vaccine influences the hemagglutinin activity of *P. gingivalis*, a hemagglutination assay was performed using the serum from immunized mice with the highest ELISA titer. The hemagglutination titers of *P. gingivalis* ATCC 33277 reacting with sera from immunized mice and nonimmunized mice were 1,280 and 160, respectively (Fig. 5). The sera from the *rgpA* DNA vaccine-immunized mice had an enhanced ability to inhibit agglutination of erythrocytes. The other sera from *rgpA* DNA vaccine-immunized mice demonstrated similar inhibitory activities against hemagglutination (data not shown).

Protection against *P. gingivalis* challenge. To evaluate the protective effect of the *rgpA* DNA vaccine, we compared the lesion sizes produced by an invasive *P. gingivalis* W50 infection in immunized and nonimmunized mice. The mice were challenged subcutaneously with 10^9 viable cells of *P. gingivalis* W50. The mice (10 per group) were monitored for the next 5 days for the development of lesions (Fig. 6). Although all of the mice developed lesions when challenged with *P. gingivalis* strain W50, the mice which had received the *rgpA* DNA vaccine were found to have significantly smaller lesions than those of nonimmunized control groups from day 2 to day 5.

DISCUSSION

Human periodontitis is thought to be initiated by a group of gram-negative anaerobic bacteria, of which a principal organism is *P. gingivalis*. Considerable scrutiny is required to select useful immunogens that can elicit functional protection against periodontal tissue destruction induced by oral microorganisms. A previous report has established that inhibition of the enzyme activity of the *P. gingivalis* cysteine proteinases, including Arg-gingipain, severely limits the colonization and infection capabilities of the microorganism (13). RgpA may play a central role in the pathogenesis of periodontal disease via production of pathophysiologically significant proteins. In the present study, an *rgpA* DNA vaccine was constructed in order to induce antibody-inhibitory products. Eukaryotic expression vectors carrying a cytomegalovirus promoter have been used to induce effective immune responses to antigens in vivo by Gene Gun

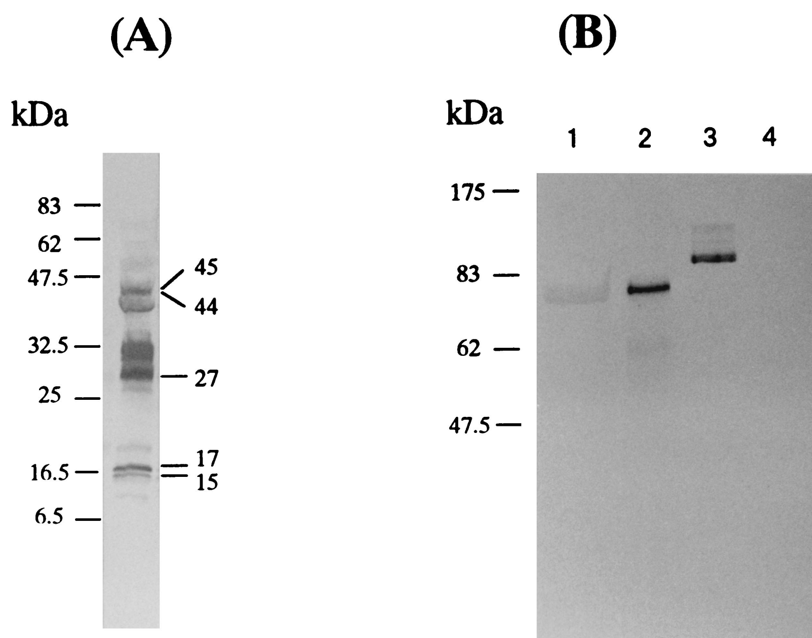

FIG. 4. Western blot analysis of sera from *rgpA* DNA vaccine-immunized mice. Sonic extracts of *P. gingivalis* or *E. coli*, which served as host cells for the expression of recombinant RgpA polypeptides (expression of the recombinant catalytic domain, HGP 44, and the HGP complex of the hemagglutinin domain), were subjected to SDS-PAGE and Western blotting. (A) Sonic extract of *P. gingivalis*. (B) Lanes: 1, recombinant catalytic domain; 2, recombinant HGP 44 of hemagglutinin domain; 3, recombinant HGP complex (HGP 15, 17, and 27); 4, BL21 (negative control).

immunization. Administration of DNA with the Gene Gun is the most efficient approach, requiring 250- to 5,000-fold less DNA than parenteral injection techniques (7, 15). This approach also offers an added safety advantage over intramuscular injection, since the administered DNA is removed from the skin through normal epidermal cell turnover (11, 28). Immunization of mice with the *rgpA* DNA vaccine resulted in expression of the mRNA of RgpA around the immunized region. The presence of *rgpA* gene products in the cells of the immunized region was demonstrated by RT-PCR. In ELISA assays, sera from mice immunized with the DNA vaccine reacted with sonic extracts of *P. gingivalis*. These results indicated that the anti-*P. gingivalis* antibody responses were generated by administration of the *rgpA* DNA vaccine using the Gene Gun.

In Western blot assays, multiple protein bands were recognized in sonic extracts of *P. gingivalis*, suggesting that the antibody reacted to degraded components of RgpA (23, 27). *P. gingivalis*-specific antibodies were not recognized in the nonimmunized sera (data not shown). Therefore, these multiple protein bands corresponded to the 45-kDa catalytic domain and the 44-kDa HGP 44, 27-kDa HGP 27, 17-kDa HGP 17, and 15-kDa HGP 15 components of the hemagglutinin domain of RgpA (21). This is consistent with the results demonstrating that sera from mice immunized with this DNA vaccine reacted with the recombinant catalytic domain, recombinant HGP 44, and the recombinant HGP complex. In addition, RgpA, Kgp, and HagA are known to have highly homologous regions in their amino acid sequences. Multiple bands obtained in the present study suggested that the antibodies induced by the vaccine reacted with these heterologous molecules. Interestingly, the HGP 44 protein of the RgpA hemagglutinin domain was strongly recognized in the sera with sonic extracts of *P. gingivalis*. A recent paper reported that epitopes within the HGP 44 adhesins induced protection in both human and mouse studies (22). This fact supports the proposal that the *rgpA* DNA vaccine can induce protective antibodies. Furthermore, the present study yielded the interesting observation that the enzyme activities of the Rgp proteinases of *P. gingivalis* were inhibited by the sera from the *rgpA* DNA vaccine-immunized mice. It is known that the Rgp proteinases act as the

TABLE 2. Inhibition of *P. gingivalis* binding to the honeycomb type I collagen sponge

Serum	Mean cpm ± SD (% inhibition)[a] with strain:	
	ATCC 33277	W83
None	2,117.0 ± 355.8 (0)	480.0 ± 88.9 (0)
From non-immunized mice	1,111.0 ± 105.2 (47.5)	121.2 ± 28.4 (74.8)
From *rgpA* DNA vaccinated mice	762.5 ± 158.0 (64.0)[b]	74.1 ± 30.1 (84.6)[c]

[a] Inhibition was statistically analyzed using the Mann-Whitney U test.
[b] Significantly different from the levels for nonimmunized sera ($P < 0.005$).
[c] Significantly different from the levels for nonimmunized sera ($P < 0.05$).

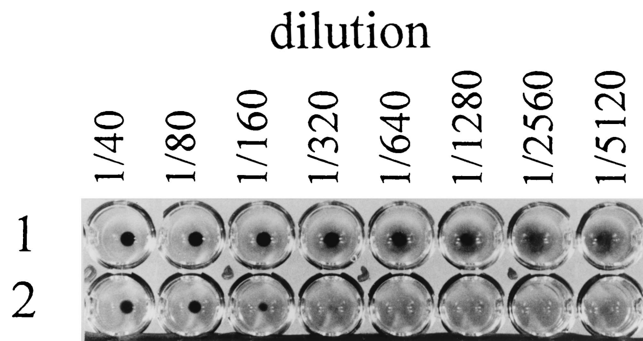

FIG. 5. Inhibition of *P. gingivalis* hemagglutination ability. The hemagglutination titers of *P. gingivalis* ATCC 33277 reacting with sera from immunized mice (1) and nonimmunized mice (2) are 1,280 and 160, respectively.

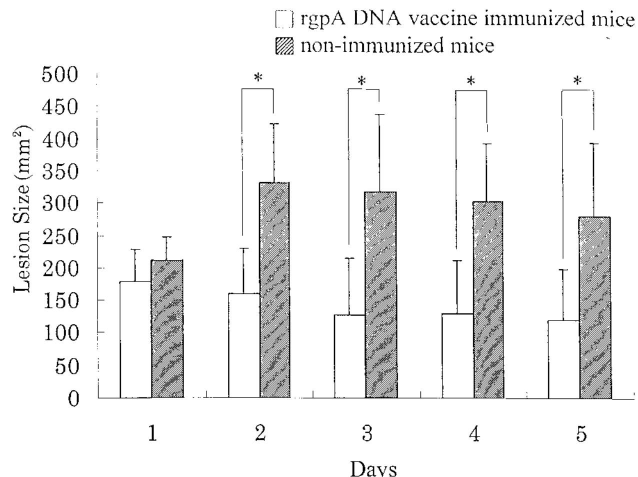

FIG. 6. Lesion sizes in mice challenged by invasive *P. gingivalis* strain W50. Lesion sizes are expressed as means ± standard deviations in square millimeters. *, significantly different by Student's *t* test ($P < 0.005$).

processing proteinase responsible for self-maturation. The Rgp proteinases play a central role in the pathogenesis of periodontal disease via production of pathophysiologically significant proteins. This study suggests that the *rgpA* DNA vaccine induces neutralizing antibody which resists the virulence potential of *P. gingivalis*.

The RgpA proteinase is involved in maintaining many of the potential colonization properties of this organism. Inactivation of the *rgpA* gene results in decreased binding to collagen (37). However, this protease defect in *P. gingivalis* results in attenuated expression of the major fimbriae, which ultimately affects the colonization properties of these organisms. In this study, binding to collagen was reduced by sera from mice immunized with the *rgpA* DNA vaccine. The proteinase may be directly associated with colonization of *P. gingivalis*, and it may reduce the ability of *P. gingivalis* to affect fimbria formation. Thus, sera from mice immunized with the *rgpA* DNA vaccine have a delayed ability to interact with matrix proteins such as type I collagen. These results suggest that the *rgpA* DNA vaccine inhibits colonization by this microorganism.

Hemagglutination on the *P. gingivalis* cell surface was first reported by Okuda et al. (24, 25). The specific genes, such as *hagA* for *P. gingivalis* hemagglutinins, which contain four repeating units, like the RgpA and Kgp repeat domains, have been isolated (31). It was suggested that the hemagglutinin-associated short motifs found in the *P. gingivalis* chromosome encode many hemagglutinin and/or hemagglutinin-related proteinases (32). A role for RgpA in *P. gingivalis* hemagglutination was indicated by the marked decrease in this activity in an *rgpA* mutant (21). Moreover, another report (2) indicated that the inhibition of hemagglutination in vivo by administration of a monoclonal antibody specific for the hemagglutinin domain of the *rgpA* gene product results in the inhibition of *P. gingivalis* colonization. In the present study, sera from mice immunized with the *rgpA* DNA vaccine reduced hemagglutinin activity by *P. gingivalis*. In addition, the hemagglutinin domain was suggested to be important for *P. gingivalis* cells to aggregate erythrocytes and bind to hemoglobin, leading to heme acquisition (1).

The present study demonstrates that immunization of mice with the *rgpA* DNA vaccine protects against challenge with invasive *P. gingivalis* strain W50 in the mouse lesion model. Immunization with the *rgpA* DNA vaccine decreased lesion size by 60% compared to the controls. This fact suggests that this vaccine could induce protective immunity against *P. gingivalis* infection. A recent report (22) showed that synthetic peptides from the RgpA catalytic and hemagglutinin domains also induced protective immunity against challenge with *P. gingivalis* in mice. Thus, RgpA is a candidate for vaccine development, and the *rgpA* DNA vaccine may be a useful protective vaccine against periodontal diseases caused by *P. gingivalis*.

In conclusion, successful expression of a target antigenic protein via a DNA vaccine using a Gene Gun was demonstrated by the presence of antibodies in immunized mice. Immunization with the *rgpA* DNA vaccine resulted in the production of antibodies which inhibited the activities of *P. gingivalis* virulence factors. These results suggest that *rgpA* is a potential candidate for the development of a DNA-based vaccine against periodontitis induced by *P. gingivalis* and indicate that Gene Gun vaccination with the *rgpA* DNA vaccine is a feasible technique. The *rgpA* DNA vaccine blocks Arg-gingipain-mediated hemagglutination and binding to collagen and possibly other Rgp-host interactions related to *P. gingivalis*-associated periodontal disease. Further studies, with primates, are in progress to examine the inhibition of *P. gingivalis* colonization and the prevention of periodontitis by *rgpA* DNA vaccine-elicited immune responses.

ACKNOWLEDGMENTS

We acknowledge H. K. Kuramitsu for thoughtful review of the manuscript. We also thank H. Kizaki, K. Minaguchi, S. Yamada, and T. Kato for helpful discussions and critical readings of the manuscript.

This work was supported by Oral Health Science Center grant 991A01 from Tokyo Dental College.

REFERENCES

1. **Barkocy-Gallagher, G. A., N. Han, J. M. Patti, J. Whitlock, A. Progulske-Fox, and M. S. Lantz.** 1996. Analysis of the *prtP* gene encoding porphypain, a cysteine proteinase of *Porphyromonas gingivalis*. J. Bacteriol. **178:**2734–2741.

2. **Curtis, M. A., J. Aduse-Opoku, J. M. Slaney, M. Rangarajan, V. Booth, J. Cridland, and P. Shepard.** 1996. Characterization of an adherence and antigenic determinant of the ArgI protease of *Porphyromonas gingivalis* which is present on multiple gene products. Infect. Immun. **64:**2532–2539.
3. **Curtis, M. A., H. K. Kuramitsu, M. Lantz, F. L. Macrina, K. Nakayama, J. Potemopa, E. C. Reynolds, J. Aduse-Opoku.** 1999. Molecular genetics and nomenclature of proteases of *Porphyromonas gingivalis*. J. Periodontal Res. **34:**464–472.
4. **Cutler, C. W., J. R. Kalmer, and C. A. Genco.** 1995. Pathogenic strategies of the oral anaerobe *Porphyromonas gingivalis*. Trends Microbiol. **3:**45–51.
5. **Davis, H. L., B. A. Demeneix, B. Quantin, J. Coulombe, and R. G. Whalen.** 1993. Plasmid DNA is superior to viral vectors for direct gene transfer into adult mouse skeletal muscle. Hum. Gene Ther. **4:**733–740.
6. **Doelly, J. J., J. B. Ulmer, J. W. Shiver, and M. A. Liu.** 1994. Immunization with DNA. J. Immunol. Methods **176:**145–152.
7. **Doelly, J. J., J. B. Ulmer, J. W. Shiver, and M. A. Liu.** 1997. DNA vaccines. Annu. Rev. Immunol. **15:**617–648.
8. **Ebersole, J. L., L. Kesavalu, S. L. Schneider, R. L. Machen, and S. C. Holt.** 1995. Comparative virulence of periodontopathogens in a mouse abscess model. Oral Dis. **1:**115–128.
9. **Fishburn, C. S., J. M. Slaney, R. J. Carman, and M. A. Curtis.** 1991. Degradation of plasma proteins by the trypsin-like enzyme of inhibition of protease activity by a serine protease inhibitor of human plasma. Oral Microbiol. Immunol. **6:**209–215.
10. **Fujimura, S., and T. Nakamura.** 1989. Multiple forms of proteases of *Bacteroides gingivalis* and their cellular location. Oral Microbiol. Immunol. **4:**227–229.
11. **Fynan, E. F., R. G. Webster, D. H. Fuller, J. R. Haynes, J. C. Santoro, and H. L. Robinson.** 1995. DNA vaccines: a novel approach to immunization. Int. J. Immunopharmacol. **17:**79–83.
12. **Genco, C. A., B. M. Odusanya, J. Potempa, J. Mikolajczyk-Pawlinska, and J. Travis.** 1998. A peptide domain on gingipain R which confers immunity against *Porphyromonas gingivalis* infection in mice. Infect. Immun. **66:**4108–4114.
13. **Genco, C. A., J. Potempa, J. Mikolajczyk-Pawlinska, and J. Travis.** 1999. Role of gingipains R in the pathogenesis of *Porphyromonas gingivalis*-mediated periodontal disease. Clin. Infect. Dis. **28:**456–465.
14. **Holt, S. C., and T. E. Bramanti.** 1991. Factors in virulence expression and their role in periodontal disease pathogenesis. Crit. Rev. Oral Biol. Med. **2:**177–281.
15. **Johnston, S. A., and D. C. Tang.** 1994. Gene Gun transfection of animal cells and genetic immunization. Methods Cell Biol. **43:**353–365.
16. **Katz, J., K. P. Black, and S. M. Michalek.** 1999. Host responses to recombinant hemagglutinin B of *Porphyromonas gingivalis* in an experimental rat model. Infect. Immun. **67:**4352–4359.
17. **Kawabata, S., Y. Terao, T. Fujiwara, I. Nakagawa, and S. Hamada.** 1999. Targeted salivary gland immunization with plasmid DNA elicits specific salivary immunoglobulin A and G antibodies and serum immunoglobulin G antibodies in mice. Infect. Immun. **67:**5863–5868.
18. **Kuttler, K. L.** 1984. Serologic and clinical responses of preimmunized, vaccinated, and previously infected cattle to challenge exposure by two different *Anaplasma marginale* isolates. Am. J. Vet. Res. **45:**2223–2226.
19. **Laemmli, U. K.** 1970. Cleavage of structure proteins during the assembly of the head of bacteriophage T4. Nature (London) **114:**305–312.
20. **Mayrand, D., and S. C. Holt.** 1988. Biology of asaccharolytic black-pigmented *Bacteroides* species. Microbiol. Rev. **52:**134–152.
21. **Nakayama, K., T. Kadowaki, K. Okamoto, and K. Yamamoto.** 1995. Construction and characterization of arginine-specific cysteine proteinase (Arg-gingipain)-deficient mutants of *Porphyromonas gingivalis*: evidence for significant contribution of Arg-gingipain to virulence. J. Biol. Chem. **270:** 23619–23626.
22. **O'Brien-Simpson, N. M., R. A. Paolini, and E. C. Reynolds.** 2000. RgpA-Kgp peptide-based immunogens provide protection against *Porphyromonas gingivalis* challenge in a murine lesion model. Infect. Immun. **68:**4055–4063.
23. **Okamoto, K., Y. Misumi, T. Kadowaki, M. Yoneda, K. Yamamoto, and Y. Ikehara.** 1995. Structural characterization of arg-gingipain, a novel arg-gingipain-specific cysteine proteinase as a major periodontal pathogenic factor from *Porphyromonas gingivalis*. Arch. Biochem. Biophys. **316:**917–925.
24. **Okuda, K., J. Slots, and R. J. Genco.** 1981. *Bacteroides gingivalis, Bacteroides asaccharolyticus*, and *Bacteroides melaninogenicus* subspecies: cell surface morphology and adherence to erythrocytes and human buccal epithelial cells. Curr. Microbiol. **6:**7–12.
25. **Okuda, K., and I. Takazoe.** 1974. Hemagglutinating activity of *Bacteroides melaninogenicus*. Arch. Oral Biol. **19:**415–416.
26. **Ono, M., K. Okuda, and I. Takazoe.** 1987. Purification and characterization of a thiol-protease from *Bacteroides gingivalis* strain 381. Oral Microbiol. Immunol. **2:**77–81.
27. **Pavloff, N., J. Potempa, R. N. Pike, V. Prochazka, M. C. Kiefer, J. Travis, and P. J. Barr.** 1995. Molecular cloning and structural characterization of the Arg-gingipain proteinase of *Porphyromonas gingivalis*. J. Biol. Chem. **270:** 1007–1010.
28. **Pertmer, T. M., M. D. Eisenbraun, D. McCabe, S. K. Prayaga, D. F. Fuller, and J. R. Haynes.** 1995. Gene gun-based nucleic acid immunization: elicitation of humoral and cytotoxic T lymphocyte responses following epidermal delivery of nanogram quantities of DNA. Vaccine **13:**1427–1430.
29. **Potempa, J., N. Pavloff, and J. Travis.** 1995. *Porphyromonas gingivalis*: a proteinase/gene accounting audit. Trends Microbiol. **3:**430–434.
30. **Potempa, J., R. Pike, and J. Travis.** 1995. The multiple forms of trypsin-like activity present in various strains of *Porphyromonas gingivalis* are due to the presence of either Arg-gingipain or Lys-gingipain. Infect. Immun. **63:**1176–1182.
31. **Seok-Woo, L., J. D. Hillman, and A. Progulske-Fox.** 1996. The hemagglutinin genes *hagB* and *hagC* of *Porphyromonas gingivalis* are transcribed in vivo and shown by use of a new expression vector. Infect. Immun. **64:**4802–4810.
32. **Shibata, Y., M. Hayakawa, H. Takiguchi, T. Shiroza, and Y. Abiko.** 1999. Determination and characterization of the hemagglutinin-associated short motifs found in *Porphyromonas gingivalis* multiple gene products. J. Biol. Chem. **274:**5012–5020.
33. **Slots, J., and R. J. Genco.** 1984. 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.
34. **Smalley, J. W., and A. J. Birss.** 1991. Extracellular vesicle-associated and soluble trypsin-like enzyme fractions of *Porphyromonas gingivalis* W50. Oral Microbiol. Immunol. **6:**202–208.
35. **Sojar, H. T., N. Hamada, and R. J. Genco.** 1997. Isolation and characterization of fimbriae from a sparsely fimbriated strain of *Porphyromonas gingivalis*. Appl. Environ. Microbiol. **63:**2318–2323.
36. **Tokuda, M., M. Duncan, M. I. Cho, and H. K. Kuramitsu.** 1996. Role of *Porphyromonas gingivalis* protease activity in colonization of oral surfaces. Infect. Immun. **64:**4067–4073.
37. **Tokuda, M., T. Karunakaran, M. Duncan, N. Hamada, and H. K. Kuramitsu.** 1998. Role of Arg-gingipain A in virulence of *Porphyromonas gingivalis*. Infect. Immun. **66:**1159–1166.
38. **Travis, J., J. Potempa, and H. Maeda.** 1995. Are bacterial proteinases pathogenic factors? Trends Microbiol. **3:**405–407.
39. **Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. J. Dwarki, S. H. Gromkowski, R. R. Deck, C. M. DeWitt, A. Friedman, L. A. Hawe, K. R. Leander, D. Martines, H. C. Perry, J. W. Shiver, D. L. Montgomery, and M. A. Liu.** 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science **259:**1745–1749.
40. **Wolff, J. A., R. W. Malone, P. Williams, G. Chong, G. Acsadi, A. Jani, and P. L. Felgner.** 1990. Direct gene transfer into mouse muscle in vivo. Science **247:**1465–1468.

Editor: D. L. Burns