Oral Microbiology Immunology 2004: 19: 77–82
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Oral Microbiology and Immunology

Expression of the β-adhesin part of HRgpA in Sprague Dawley rats induces a specific antibody response

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Vågnes KS, Vågnes ØB, Vasstrand EN, Bakken V. Expression of the β-adhesin part of HRgpA in Sprague Dawley rats induces a specific antibody response. Oral Microbiol Immunol 2004: 19: 77–82. © Blackwell Munksgaard, 2004.

The β-adhesin part of the *Porphyromonas gingivalis* W50 (ATCC 53978) protease HRgpA was cloned in an eukaryotic expression vector and expressed in COS-7 cells. The monoclonal antibody MAb (61BG1.3), specific for the hemagglutinating domain of β-adhesin, recognized the expressed β-adhesin in the transfected cells both by immunoblot and immunofluorescence. Sprague Dawley rats were immunized intramuscularly with β-adhesin encoding expression plasmid and expression plasmid without β-adhesin insert. Skeletal muscle tissue at the site of immunization in the β-adhesin immunized animals was shown to express this protein. The immunization induced a β-adhesin-specific antibody response. Sera from the immunized animals were tested for hemagglutination inhibiting activity. Due to high natural inhibiting activity in all rat sera tested, no increased hemagglutination inhibition was detected in sera from the β-adhesin immunized animals.

Key words: *Porphyromonas gingivalis*; HRgpA; nucleic acid immunization; immune response

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Accepted for publication September 22, 2003

Periodontitis is an inflammatory disease leading to degradation of dental supportive tissue and, in the terminal stage, dental loss. The disease is of ancient origin. In Britain the prevalence has been shown to be unchanged for the past 3000 years (19). Epidemiologic studies from the western hemisphere show that 30–40% of the dentate population is affected in some way by periodontitis, with about 13% seriously being affected (16, 26).

Periodontitis is associated with an increase in several anaerobic bacteria, with *Porphyromonas gingivalis* the one most commonly associated with adult periodontitis (13, 33). This is a gram-negative asaccharolytic bacterium with an obligate need of iron. Iron is acquired in the form of hemin from hemolyzed erythrocytes due to the lack of a siderophore system (5, 17). The hemagglutination is important for the ability to achieve hemin. The strong hemagglutinating activity depends on several hemagglutinating proteins. At least five hemagglutinins (Hag A, Hag B, Hag, C, Hag D, Hag E) and two proteases, arginine-specific hetereodimeric protease (HrgpA) and lysine specific protease (Kgp), with a hemagglutinating adhesin part, have been described in *P. gingivalis* (2, 7, 14, 23, 24, 28, 29). The first hemagglutinin to be sequenced, Hag A, was shown to contain four repeating elements (14). Parts of these elements have later been found in Hag D, Hag E and the two hemagglutinating proteases (2). Monoclonal antibodies inhibiting *P. gingivalis* hemagglutination were shown to bind in a part of these elements containing the sequence PVQNLT (9, 18, 32). Shibata et al. also identified PVQNLT as the smallest hemagglutinating motif in these hemagglutinins (32). Booth et al. (4) used a monoclonal antibody (3) against this epitope for passive immunization of periodontitis patients who had gone through professional dental prophylaxis. In these patients, re-colonization of *P. gingivalis* was inhibited for up to 9 months after immunization. Proteins containing the PVQNLT epitope are therefore possible candidates for a vaccine against *P. gingivalis*.

The proteolytic activity is a main pathogenic factor for the bacteria. The arginine-specific protease HRgpA is one of the most important proteases. As mentioned above, this protease also contains a hemagglutinating β-adhesin part. In addition to the hemagglutination activity, the β-adhesin also binds specifically to fibronectin in the extracellular matrix. Due to this, HRgpA has a 12-fold higher proteolytic activity of fibronectin than the non β-adhesin-containing protease RgpB (30). The importance

of β-adhesin for both bacterial hemagglutination and proteolytic activity further supports the use of this protein as a vaccine against *P. gingivalis*.

P. gingivalis has been proposed to invade gingival epithelial cells in tissue both from patients with adult periodontitis and healthy subjects (21, 22). In addition, nuclear entry of HRgpA has been observed with cell lines of both oral and non-oral origin (31). An intracellular location may therefore be a common ecologic niche for *P. gingivalis* in both health and disease, both as an infectious portal and for evading a host immune response (22). Bacterial vaccines are usually proteins and/or polysaccharides from whole cells or subcellular components inducing a Th2 antibody response with production of IgG1 and IgG3 in human and mouse, and IgG1 and IgG2a in rat. The only conventional vaccines inducing an immune response that are also effective against intracellular agents have so far been vaccines based on attenuated intracellular agents. In the past decade, a new type of immunization based on direct injection in tissue of nucleic acid encoding the protein antigen has been much studied. Nucleic acid immunization has been shown to induce both humoral and cellular immune responses and protective immunity against infectious diseases in animal models (6, 25). The endogenous expression of proteins, encoded by the DNA, results in an antigenic presentation on MHCI, facilitating the generation of a Th1-dominated immune reaction with antibody-isotypes related to this kind of reaction (12). Nucleic acid immunization is shown to be a very powerful technique that makes it possible to study immune reactions against different protein antigens without having to do pre-immunization protein expression and purification.

The purpose of the present study was to investigate whether the β-adhesin part of HRgpA from *P. gingivalis* was expressed and able to fold properly in eukaryotic cell culture. Furthermore, we examined the ability of this protein to elicit a specific anti-β-adhesin response when expressed in rat muscle tissue.

Methods

Bacterial strain and culture conditions

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

Construction of plasmid DNA for immunization

Genomic DNA from *P. gingivalis* W50 was isolated by phenol chloroform extraction from cultivated bacteria. Primers were constructed to amplify a 1164-bp fragment and a 864-bp fragment of the β-adhesin part of the *rgpA* gene. The PCR primers used were as follows; for fragment 1 (1164 bp) the sense primer was 5′-AATATGGGTCCG-AACTGTAGTGTC-3′ and for fragment 2 (864 bp) the sense primer was 5′-ATAA-TGGAAGGTGGTGGAAGCGATTAC-3′. The antisense primer 5′-CTCGAACGTT-TCCGTGAAGTCTGCGCG-3′ was used for both fragments. A translation initiation sequence, ANNATGG, was included at the 5′ ends in both sense primers. The β-adhesin fragments were amplified using AmpliTaq Gold (Applied Biosystems, Foster City, CA) by parameters as follows: denaturation at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 1 min; annealing at 55°C for 1 min; and extension at 72°C for 1 min. This was followed by final extension at 72°C for 10 min. The amplified β-adhesin fragments were inserted into the eukaryotic expression vector pcDNA3.1/V5-His-TOPO (Invitrogen, Carlsbad, CA). The sequence of the cloned DNA was checked by sequencing (Big Dye Cycle Sequencing Kit, Applied Biosystems). Based on the sequencing results, two clones of fragment 1 and one clone of fragment 2 were selected for use in expression and immunization.

Expression of β-adhesin in cell culture

COS-7 cells were grown on coverslips in 24-well plates with IMDM medium (Iscoves Modified Dulbeccos Modified Minimum Essential Medium, Bio Whittaker, Cambrex bioscience, Verviers, Belgium) supplemented with 5% fetal calf serum, 1% glutamine, 1% amphotericin, 1% streptomycin and 1% penicillin. The cells were transfected with two different clones of the 1164 bp fragment (clone 1.10 and 1.13) and one clone of the 864 bp fragment (clone 2.1) using Fugene 6 (Boehringer Mannheim, Mannheim, Germany) as described by the manufacturer.

Immunofluorescence

Twenty-four hours after transfection the cells were fixed with 4% paraformaldehyde for 20 min, washed twice with PBS and incubated overnight with methanol at −20°C. The coverslips containing the transfected cells were then washed 3 times in phosphate-buffered solution (PBS), blocked for 15 min with 0.5% bovine serum albumin (BSA) in PBS at room temperature and thereafter incubated at 4°C overnight in a humidity chamber with a β-adhesin specific monoclonal antibody, MAb 61BG3.1 (kindly provided by Dr. Rudolf Gmür, University of Zürich, Switzerland). The coverslips were then washed three times in PBS, incubated for 1 h at room temperature with fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG1 Ab (Southern Biotechnology, Birmingham, AL) diluted 1:50 in blocking solution. Finally, the coverslips were washed three times and mounted on Slow Fade.

Immunoblot analysis

Forty-eight hours after transfection, as described above, the COS-7 cells were incubated with 150 μl hypotone PBS at 4°C for 5 min and then 50 μl 4× sample buffer containing mercaptoethanol was added for sodium dodecyl sulfate polyacrylamide gel-electrophoresis (SDS–PAGE) (20). The samples were then heated at 95°C for 5 min and loaded on a SDS–PAGE gel (4 and 12% acrylamide in stacking and separation gel, respectively). After separation the proteins were transferred to a nitrocellulose membrane by electrophoresis at 4°C, 100 V for 30 min. After this the membrane was washed twice with Tris-buffered saline (TBS) and blocked 1 h with 3% dried skimmed milk diluted in TBS. The membrane was then washed 2 × 5 min and 1 × 15 min with TBS-Tween (0.05%) and incubated 1 h with primary Ab (MAb 61BG1.3) diluted 1:200 in 0.5% dried skimmed milk in TBS-Tween. The membrane was then washed as above and incubated for 1 h with peroxidase-conjugated anti-mouse secondary antibody (Amersham Pharmacia, Uppsala, Sweden) diluted 1:1000 in 0.5% dried skimmed milk in TBS-Tween. Finally, the membrane was washed 1 × 15 min with TBS-Tween and 2 × 5 min with TBS. The immune reaction was detected using the ECL-detection reagent (Amersham Pharmacia) as described by the manufacturer.

Rat immunization

The DNA immunization was done in male Sprague Dawley rats (Møllegård, Skensved, Denmark) fed on standard rat chew and kept in groups of four animals. At the time of the first immunization the rats were 8 weeks old. Four groups containing four animals were used. Each group was immunized with clone 1.10, clone 1.13

(large fragment), clone 2.1 (small fragment) or pcDNA3.1 without any insert. Each animal was immunized intramuscularly in the femur with 100 μg DNA solved in 100 μl PBS containing 25% sucrose. The animals were immunized 3 times at 4-week intervals. They were sacrificed 10 days after the last immunization and blood and femural muscle tissue collected.

Detection of *P. gingivalis* β-adhesin mRNA in immunized animals

Femoral skeletal muscle was dissected from the immunized animals. The muscle tissue was homogenized and total RNA extracted and isolated by the use of TRIzol® (Gibco, Invitrogen, Carlsbad, CA) following the procedure described by the manufacturer. Total RNA 1 μg was amplified by reverse transcriptase polymerase chain reaction (RT-PCR) using an Access RT-PCR kit (Promega, Madison, WI). The primers used for the RT-PCR were 5'-GTATGCGCAC-AGGATGCTAA-3' (sense) and 5'-TATT-TCGTACCTGCGGGAAG-3', resulting in a product of 200 base pairs. Reverse transcription was performed at 48°C for 45 min. Amplification was performed with 40 cycles of 94°C for 1 min, 57°C for 30 s and 68°C for 30 s. This was followed by a final elongation period of 72°C for 10 min. The amplification product was displayed on an ethidium bromide-stained 2% agarose gel.

Enzyme linked immunosorbent assay (ELISA)

P. gingivalis bacteria were grown as described above, washed 3 times in PBS and sonicated three times for 30 s on ice. A 96-well Maxi Sorp plate (Nunc, Roskilde, Denmark) was coated with bacteria, 40 μg bacterial sonicate in 100 μl 50 mM carbonate buffer per well, and incubated at 4°C overnight. The wells were blocked with 3% skimmed milk in PBS and incubated at room temperature for 1 h, 100 μl of twofold serial dilutions of rat serum in PBS were incubated at room temperature for 1 h, and 100 μl alkaline phosphatase-conjugated goat anti-rat total IgG antibody (Sigma, St Louis, MO) was then added and incubated at room temperature for 1 h. The wells were washed three times with PBS-Tween after each step. Substrate was added and absorbance measured at λ_{405nm}. The serum dilution resulting in an $A_{405nm} \geq 0.2$ was used as end point titer.

For *P. gingivalis*-specific IgG subclass detection, Maxi Sorp wells were coated with bacteria and blocked with dried skimmed milk as described above. Twofold dilutions of pooled rat sera were then incubated and ELISA performed as described. For total IgG subclass detection, Maxi Sorp wells were coated directly with twofold dilutions (1:100–1:25000) of pooled sera from immunized and non-immunized rats. ELISA was then performed. The rat sera concentrations resulting in an absorbance (λ_{405nm}) of 0.8 were chosen, and new ELISA tests for total IgG and *P gingivalis*-specific IgG subclass detection were performed. The amount of rat IgG was identical in all three tests. Mouse anti-rat IgG1 (diluted 1:500), mouse anti-rat IgG2a (diluted 1:5000), mouse anti-rat IgG2b (diluted 1:1000) (all Sigma antisera) were used as secondary antibody. Mouse sera were then incubated with alkaline phosphatase-conjugated rabbit anti-mouse (diluted 1:1000) (Dako, Glostrup, Denmark). All antibody incubations were performed at room temperature for 1 h followed by washing with PBS-Tween. Substrate was added and absorbance measured at λ_{405nm}. The absorbance ratio of the subclass detections was used to describe the subclass tendencies.

Hemagglutination

Overnight cultures of *P. gingivalis* W50 (ATCC 53978) were centrifuged, washed 3 times with PBS, and resuspended in PBS. A dilution series of the resuspended bacteria from $OD_{600nm} = 2$ to $OD_{600nm} = 0.02$ was made for determination of the bacterial concentration necessary for hemagglutinating activity in sheep erythrocyte suspension (2% in PBS). Aliquots of 25 μl of each bacterial dilution were mixed with equal volumes of PBS and sheep erythrocyte suspension and incubated at room temperature for 180 min in a 96-well round-bottomed microtiter plate. The highest bacterial dilution still exhibiting full agglutination was used in hemagglutination inhibition tests. Sera from Sprague Dawley rats immunized with β-adhesin coding plasmid and non coding control plasmid, pooled normal sera from non immunized Sprague Dawley rats (10 animals), human sera from two person without periodontitis, and commercial normal sera from pig and mouse (Dako) were used in the assay. The sera were diluted in twofold series with PBS. Aliquots of 25 μl of each dilution were mixed with equal volumes of the bacterial suspension, added to a round-bottomed microtiter plate and incubated at room temperature for 30 min. After incubation, 25 μl of 2% erythrocyte suspension was added to each well, and incubated at room temperature for 180 min. The wells were then inspected for hemagglutinating activity. The same procedure was also applied to heat-inactivated sera (56°C for 30 min).

Results

Sequencing of cloned fragments

The sequencing results from clone 2.1 (864-bp fragment) and from clones 1.10 and 1.13 (1164-bp fragment) were homologous except from the PN (proline/asparagine) rich sequence found in clone 2.1 and 1.13, but not in clone 1.10. However, BLAST search with both the PN-rich and PN-missing sequences showed homology with earlier sequencing results.

Immunofluorescence

Indirect immunofluorescence was positive for all three clones (2.1, 1.10, 1.13). However, the portion of transfected cells and the amount of adhesin expression was higher in cells transfected with clone 2.1 than in cells transfected with clones 1.10 and 1.13. The protein was evenly distributed in the cytoplasm and nucleus of the cells, but was absent from the nucleolus (Fig. 1).

Immunoblot analysis

The ECL immunoblot (Fig. 2) showed several bands in the sample containing *P. gingivalis* lysate, and three bands (37.5, 38 and 60 kDa) in the sample containing Cos-7 cells transfected with clone 2.1.

Detection of *P. gingivalis* β-adhesin mRNA in immunized animals

To check for β-adhesin gene expression in the immunized animals, femoral muscle

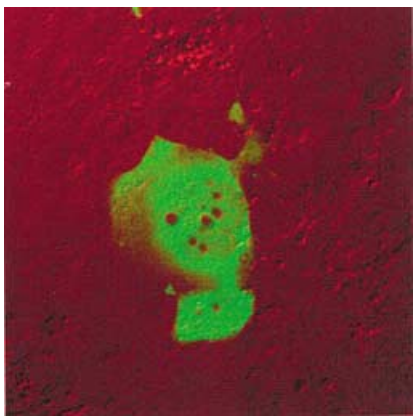

Fig. 1. Confocal microscopy of cells transfected with clone 2.1, incubated with Mab 61BG1.3 and FITC-conjugated secondary antibody. The expressed adhesin is visualized as fluorescent green in the cell cytoplasm and nucleus.

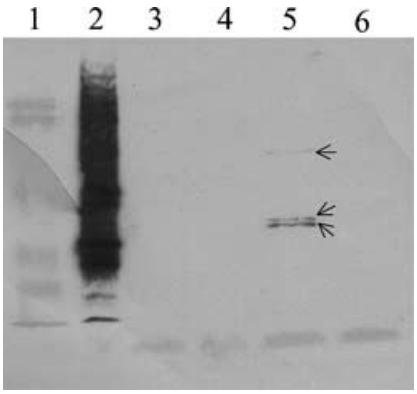

Fig. 2. Immunoblot with Low Range Prestained SDS–PAGE molecular weight standard (BIO-RAD) (well 1), *P. gingivalis* lysate (well 2), clone 1.10 (well 3), clone 1.13 (well 4), clone 2.1 (well 5) and Cos7 cells (well 6). Arrows indicate three protein bands with molecular weights (from bottom) of 37.5 kDa, 38 kDa and 60 kDa.

tissue was tested by RT-PCR for β-adhesin mRNA. *P. gingivalis* β-adhesin mRNA was amplified from all β-adhesin immunized animals. The test results from pcDNA 3.1 immunized animals were negative.

Detection of anti-adhesin antibodies in rat serum

The titers of anti-adhesin antibodies were measured by ELISA of sera from rats immunized with the DNA vaccine plasmids (2.1, 1.10 and 1.13) or with the expression vector alone (pcDNA3.1). Specific antibodies against *P. gingivalis* were detected in all sera from rats immunized with the vaccine plasmids, but not in sera from rats immunized with the control plasmid (Fig. 3). There was an increased amount of total sera IgG2b in pooled sera from β-adhesin-immunized rats compared to pooled sera from pcDNA3.1-immunized rats (Table 1). Also the *P. gingivalis*-specific IgG antibodies had an increased

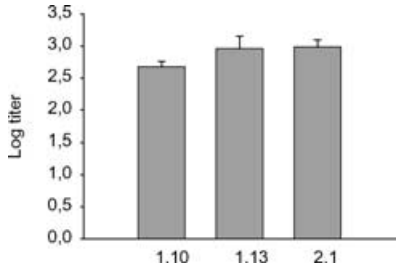

Fig. 3. P. gingivalis-specific antibody titers in sera from the different groups of immunized rats. Serial dilutions were made until $A_{405nm} \geq 0.2$. Values are presented as means ± standard error of log ELISA antibody titer.

Table 1. Ratios[1] of IgG2b/IgG2a and IgG2b/IgG1

	IgG2b/IgG2a	IgG2b/IgG1
Pooled pcDNA3.1 immunized sera total IgG	0.24	2.54
Pooled β-adhesin immunized rat sera total IgG	0.48	3.78
Pooled *P. gingivalis*-specific IgG	0.58	3.14

[1]Absorbance measured at $\lambda = 405$ nm.

amount of IgG2b compared to the total IgG subclass distribution of sera from pcDNA3.1-immunized animals.

Serum-inhibition of hemagglutinating activity of *P. gingivalis*

All rat sera inhibited hemagglutination until a serum dilution of 1:1024 with individual sera inhibiting up to a 1:2048 dilution. There was no difference between immunized and non-immunized animals. Control sera (human, mouse, and pig) clearly inhibited the hemagglutinating activity up to a 1:512 dilution. Similar results were obtained with heat-inactivated sera. There was no connection between the response obtained with ELISA and hemagglutination inhibition.

Discussion

We have shown that the β-adhesin part of HRgpA can be expressed in eukaryotic cells both in cell culture and *in vivo* in rat femoral muscle. *In vivo* expressed protein β-adhesin is able to elicit the specific antibody response.

The monoclonal antibody 61BG1.3 has a neutralizing effect on *P. gingivalis* (4), and has been shown to inhibit recolonization of the bacterium when used in passive immunization of periodontitis patients. The epitope of this antibody is localized to the peptide motif GVSPKVCKDVTVEGSNEFAPVQNLT (18). The PVQNLT motif is found in several hemagglutinins of the bacterium (A, D and E) and in the lysine-cysteine protease (Kgp), and the β-adhesin part of the arginine-cysteine protease (HRgpA). PVQNLT has been shown to be associated with the hemagglutinating activity of the bacterium (32). This shows a great similarity with the domain PLQNLT in the influenza virus A hemagglutinin HA1 (10).

In this study we have shown that the 61BG1.3 antibody is able to recognize the expressed β-adhesin protein from the three clones (clones 2.1, 1.10, 1.13) in eukaryotic cell culture. However, cells transfected with clone 2.1 had a greater percentage of successfully transfected cells, and more intense fluorescence when viewed in the fluorescence microscope than cells transfected with clones 1.10 and 1.13. One reason for this might be reduced antibody recognition due to misfolding of the β-adhesin expressed in cells transfected with clones 1.10 and 1.13. Another reason could be differences in the protein expression due to the existence of cryptic inhibitors in the long fragments (1.10, 1.13). Reduced protein expression has previously been connected with cryptic transcription inhibitors (15). The lack of the PN-rich sequence in fragment 1.10 had no detectable effects on the expression level.

The expression pattern found by the fluorescence technique was confirmed in the ECL-immunodetection assay. The protein expression in cells transfected with clones 1.10 and 1.13 was not detectable at all, whereas the well with cells transfected with clone 2.1 showed two small bands (37.5 kDa and 38 kDa) and one large band (60 kDa). The small bands are very close to the theoretical molecular weight of 36.79 kDa, including 5 kDa encoded by the V5 epitope and His-tag regions in the pcDNA3.1/V5-His-TOPO plasmid. Predict protein analysis of the β-adhesin revaled several seats for post-translation processing like N-glycosylation and phosphorylation. This might explain the observed 38 kDa band. The weak 60 kDa band might be due to a duplication of the protein.

The distribution of the adhesin in both cytoplasm and nucleus but not nucleolus is in accordance with the observation of Scragg et al. (31). Purified heterodimeric HRgpA and recombinant adhesin β chain freely entered the cells and were found in both cytoplasm and nucleus. Recombinant catalytic α-chain freely entered the cell, but was only detected in the cytoplasmic part. The β-adhesin therefore seems to target the protease in to the nucleus. It is interesting to observe that the β-adhesin protein synthesized in the transfected cells was transported in the same manner as protein added in the cell culture.

Specific anti-adhesin antibodies were detected in all sera from rats immunized with the different DNA vaccine plasmids (clones 1.10,1.13, 2.1). The antibody titer was equal between the three different groups in spite of the better results obtained with clone 2.1 in the *in vitro* experiments. The hemagglutinating adhesin part of HRgpA and Kgp is the immunodominant protein in both human and

mice positive for *P. gingivalis* (8, 9, 11). The repeating PN sequence lacking in clone 1.10, is polar and probably exposed on the outside of the bacteria. The region containing this sequence is shown to be part of the β-adhesin most frequently recognized by sera from periodontitis patients (18). This makes this sequence the most immunodominant domain of *P. gingivalis.* However, the lack of this sequence did not influence the level of specific antibodies in the vaccinated animals.

Pooled sera from β-adhesin-immunized animals showed an increased concentration of IgG2b compared to pooled sera from pcDNA3.1 immunized animals both for total IgG and for *P. gingivalis*-specific IgG. Rat IgG2b indicates a Th1-driven immune reaction. This is in agreement with previous studies showing that intramuscular DNA immunization usually creates a Th1-dominated immune reaction (27). Since both the total IgG and the *P. gingivalis*-specific IgG populations have an increased amount of IgG2b, the whole immunologic environment in the β-adhesin-immunized rats seems to be directed in a more Th1 manner than in the pcDNA 3.1 immunized rats.

The neutralizing of *P. gingivalis* hemagglutinating activity was not enhanced in sera from the β-adhesin-immunized rats compared to sera from rats immunized with control plasmid and pooled rat normal sera. All rat sera tested, both from immunized and non-immunized animals, had a strong inhibitory effect on the hemagglutination, ranging from sera dilutions of 1:1024 to dilutions of 1:2048. This result is inconsistent with the results obtained by Yonezawa et al. (35), who found that the hemagglutination titers of *P. gingivalis* ATCC 33277 reacting with sera from immunized mice and non-immunized mice were 1:1280 and 1:160, respectively. The use of different bacterial strains could be one reason for this difference in hemagglutination inhibition. Bacterial cells of the W50 strain have previously been described to have less hemagglutinating activity compared to ATCC 33277 (34). Due to the high inhibitory activity of the sera from non-immunized rats, sera from non-immunized individuals of other mammalian species were used in the same assay. All sera tested had less inhibitory activity than rat sera (1:512 dilution). The hemagglutination inhibition assay was repeated with heat-inactivated sera. The rat sera seem to have a strong heat stable non-antibody hemagglutination-inhibiting factor. Species differences in serum inhibition of Rgp protease activity have already been described (1). Bactericidal effects on *P. gingivalis* of rat serum cystatin S have also previously been shown. Due to high natural hemagglutination inhibiting activity of rat sera we were not able to show any immunization-related differences in the inhibiting ability.

Acknowledgment

This study was supported in part by a grant from L. Meltzers høyskolefond.

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