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Short communication

Recognition and phagocytosis of multiple periodontopathogenic bacteria by anti-*Porphyromonas gingivalis* heat-shock protein 60 antisera

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*Choi J-I, Choi K-S, Yi N-N, Kim U-S, Choi J-S, Kim S-J. Recognition and phagocytosis of multiple periodontopathogenic bacteria by anti-*Porphyromonas gingivalis *heat-shock protein 60 antisera.*
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The present study has been performed to evaluate *Porphyromonas gingivalis* heat shock protein (HSP) 60 as a candidate vaccine to protect against multiple putative periodontopathic bacteria. Mouse anti-*P. gingivalis* HSP antisera demonstrated the elevated IgG antibody titers against the multiple bacteria tested and cross-reacted with heat-induced bacterial proteins of the target bacteria. The antisera also demonstrated a significantly higher opsonophagocytosis function against all the target bacteria than the control sera ($P < 0.01$). We concluded that *P. gingivalis* HSP 60 could potentially be developed as a vaccine against multiple periodontopathic bacteria.

Key words: heat shock protein; *Porphyromonas gingivalis*; periodontal disease; vaccine

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Animal experiments strongly suggest that the severity of periodontitis induced by various bacteria can be minimized by immunization with whole bacterial cells or antigens from the homologous bacteria (2, 11, 16). However, several issues should be addressed pertinent to the development of a sophisticated vaccine against human periodontitis (10, 14). First, human periodontal disease is not caused by a single pathogen. Currently, seven to 10 candidate bacteria have been claimed to be the putative periodontal pathogens including *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans, Prevotella intermedia, Campylobacter rectus*, *Tannerella forsythia*, *Treponema denticola* and *Fusobacterium nucleatum* (6). The multiplicity

Table 1. Anti-*P. gingivalis* HSP60 and antibacterial IgG antibody titers of mouse immune sera from the test and the control group (mean ± SD)[a]

	Target antigens						
Group	Pg HSP60	Pg[b]	Aa	Bf	Fn	Pi	Td
Test sera[c]	445 ± 77	1761 ± 359	1215 ± 289	1184 ± 184	1557 ± 321	1240 ± 310	1002 ± 200 (heat shock)
Test sera[d]		107 ± 21	124 ± 20	101 ± 16	132 ± 27	116 ± 15	129 ± 11 (normal condition)
Control sera	104 ± 11	114 ± 8	109 ± 12	102 ± 18	113 ± 11	94 ± 17	106 ± 15

[a]Mean antibody titers and standard deviation (SD) were calculated from the test ($n = 10$) and control ($n = 10$) mice.
[b]Pg: *P. gingivalis*, Aa: *A. actinomycetemcomitans,* Bf: *B. forsythus,* Fn: *F. nucleatum,* Pi: *P. intermedia,* Td: *T. denticola.*
[c]The test group mice sera had elevated antibody titers against the target bacteria grown under heat shock conditions when compared with the control group mice sera.
[d]The test group mice sera did not show an elevated antibody response against the target bacteria grown under normal conditions when compared with the control group mice sera.

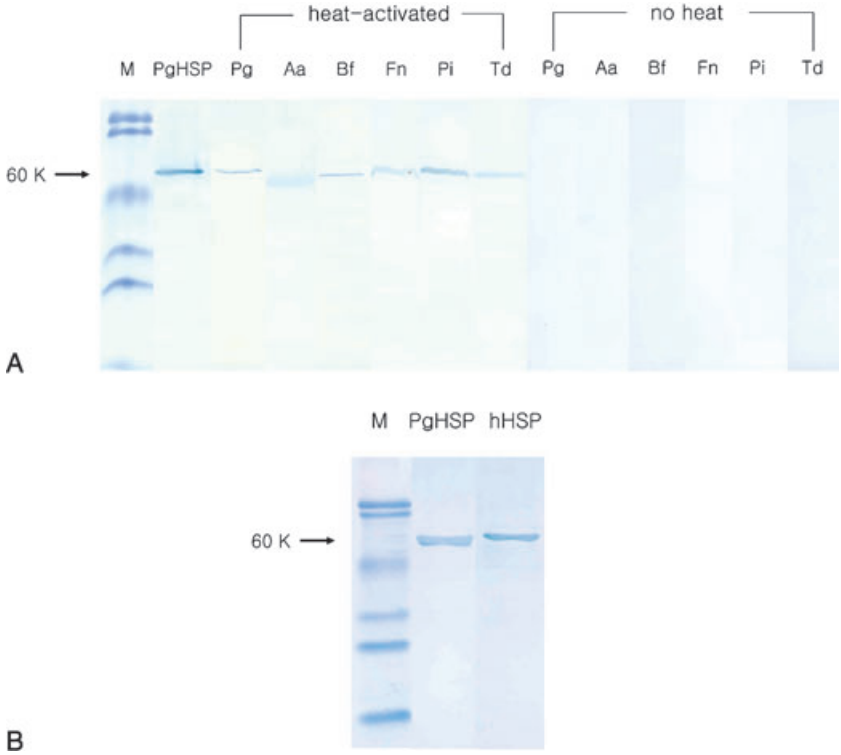

Fig. 1. Western immunoblot patterns of *P. gingivalis* HSP60 and protein extracts from heat-shocked bacteria recognized by mouse anti-*P. gingivalis* HSP60 antisera. Mouse anti-*P. gingivalis* HSP60 antisera recognized and reacted with *P. gingivalis* HSP as well as with all other HSPs induced by heat-treatment of putative periodontopathogenic bacteria tested (A, left panel). It did not react with cell lysates from bacteria grown without heat-treatment (A, right panel). Mouse anti-*P. gingivalis* HSP60 antisera also demonstrated the reactivity with homologous *P. gingivalis* HSP60 (PgHSP) and cross-reactivity with human HSP60 (hHSP) (B). 10 μg of *P. gingivalis* HSP60 and human HSP60, respectively, or 100 μg of whole cell lysates proteins of each target bacteria were loaded. Pg: *P. gingivalis*, Aa: *A. actinomycetemcomitans,* Bf: *B. forsythus,* Fn: *F. nucleatum,* Pi: *P. intermedia,* Td: *T. denticola*.

Table 2. Peak chemiluminescence intensities (mV) in opsonophagocytosis assay for each target bacteria, grown under heat shock or normal condition, using mouse anti-*P. gingivalis* HSP60 antisera group and nonimmune sera (mean ± SD)[a]

	Target bacteria grown under heat shock conditions					
	Pg [b]	Aa	Bf	Fn	Pi	Td
Test sera[c]	3632 ± 121	2088 ± 98	1521 ± 34	23,780 ± 1368	3747 ± 96	3478 ± 155
Control sera	1992 ± 56	79 ± 9	315 ± 14	7412 ± 102	2476 ± 43	2157 ± 79

	Target bacteria grown under normal conditions					
	Pg	Aa	Bf	Fn	Pi	Td
Test sera[d]	86 ± 37	613 ± 28	495 ± 53	1629 ± 166	658 ± 72	915 ± 81
Control sera	601 ± 44	589 ± 69	473 ± 31	1603 ± 79	630 ± 47	932 ± 114

[a]Mean peak intensities and standard deviation (SD) were calculated from three repeated measurements. Human peripheral blood lymphocytes were used for the opsonophagocytosis assay.
[b]Pg: *P. gingivalis*, Aa: *A. actinomycetemcomitans*, Bf: *B. forsythus*, Fn: *F. nucleatum*, Pi: *P. intermedia*, Td: *T. denticola.*
[c]All the peak chemiluminescence intensities in the test sera were significantly higher than those of the control (nonimmune) sera for all the target bacteria grown under heat shock conditions ($P < 0.01$, Student's *t*-test).
[d]None of the peak chemiluminescence intensities in the test sera was significantly different ($P > 0.05$) from those of the control (nonimmune) sera for any target bacteria grown under normal conditions.

of pathogenic organisms indicates that vaccine design against periodontitis is very complex (15). Second, bacterial whole cells or a crude extract preparation for vaccination is not desirable because with antigenic determinants of bacteria there is a potentially high risk of cross-reactivity with human counterparts. These difficulties could be overcome by a sophisticated vaccine design that incorporates well-defined antigenic epitopes or synthetic peptides unique to the virulent pathogens.

Within the context of the strategy to develop a cross-protective vaccine, we have performed a preliminary experiment utilizing *P. gingivalis* heat shock protein (HSP) as a candidate antigen for vaccine development. The rationale for selection of this bacterial antigen is that it can be found in most putative periodontopathic pathogens including *P. gingivalis* (6) and it was reported to cross-react among bacterial antigens (8).

P. gingivalis 381, *P. intermedia* 25611, *F. nucleatum* ATCC 10953, *A. actinomycetemcomitans* 33384, *T. forsythia* 43037, and *T. denticola* 33520 were grown at 37°C and subcultured at 44°C for 1 h to induce expression of HSP (the *T. denticola* 33520 culture was a gift from Dr. Ishihara, Tokyo Dental College, Chiba, Japan). Recombinant *P. gingivalis* HSP60 was purified from *Escherichia coli* transformed with *P. gingivalis* GroEL gene (13). Ten BALB/c mice were initially immunized subcutaneously with 5 μg of recombinant *P. gingivalis* HSP60 suspended in complete Freund's adjuvant followed by two subsequent subcutaneous injections of recombinant *P. gingivalis* HSP60 suspended in incomplete Freund's adjuvant at 2-week intervals. Mouse serum samples were collected by tail bleeding prior to the first immunization and 1 week after the final immunization, and stored at −20°C until used. Ten control mice were sham-immunized with complete and incomplete Freund's adjuvant, respectively. The animal experiment protocol was approved by the Institutional Review Board of Pusan National University Hospital.

Microtiter plates were coated with *P. gingivalis* HSP60 or formalinized bacterial whole cells grown either at 37°C or at 37°C followed by incubation for additional 1 h at 44°C. After washing the plates, serial dilutions of mouse serum samples were added and the plates incubated. The plates were washed and peroxidase-conjugated rabbit anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA) added. After incubation, the plates were washed followed by addition of tetramethylbenzidine (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The reaction was stopped with 0.18 M H_2SO_4. Optical densities were plotted as a function of the serum dilution factor. The best of curve fit was generated

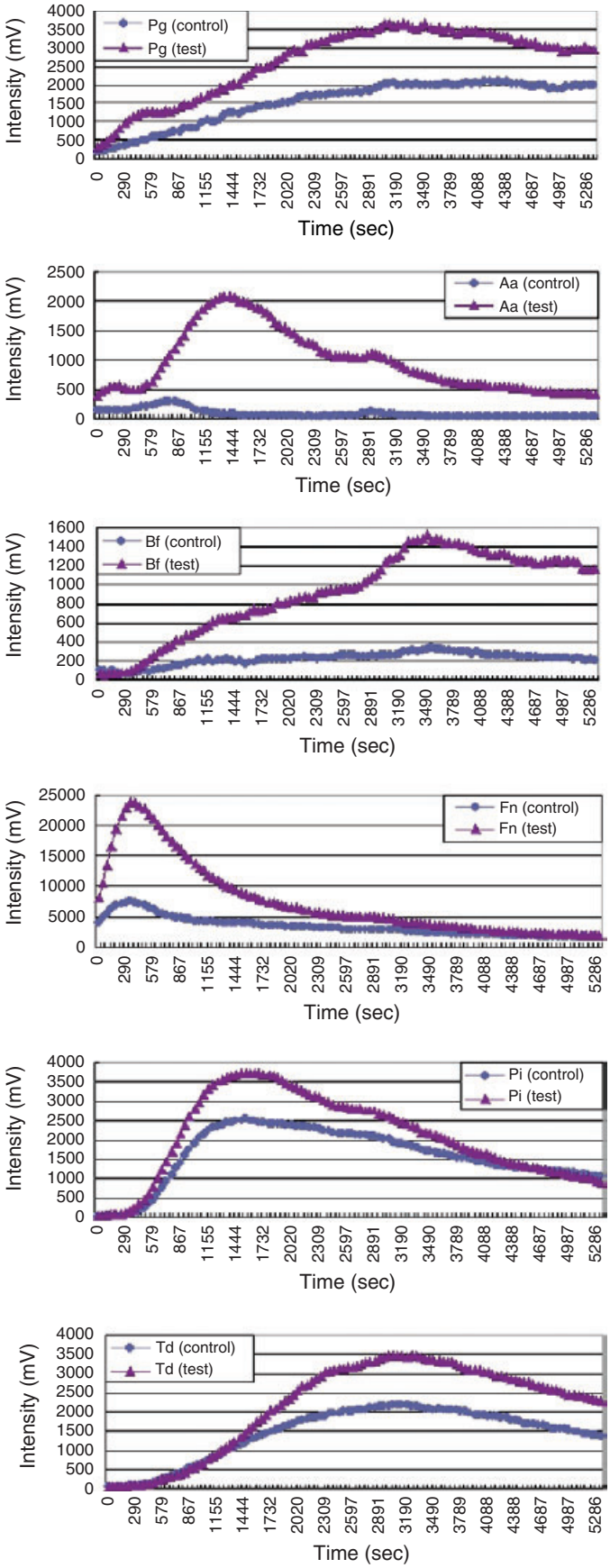

Fig. 2. Time-dependent chemiluminescence intensities (mV) for each of six target bacteria, grown under heat shock treatment, demonstrating successful opsonophagocytosis function of mouse anti-*P. gingivalis* HSP60 antisera. Polyclonal mouse anti-*P. gingivalis* HSP60 antisera yielded significantly higher time-dependent chemiluminescence intensities for all the target bacteria tested when compared with sham-immunized control sera. Td: *T. denticola,* Bf: *B. forsythus,* Aa: *A. actinomycetemcomitans,* Pg: *P. gingivalis,* Pi: *P. intermedia,* Fn: *F. nucleatum*.

and a dilution factor of each serum that gives an optical density of 0.5 was calculated for determination of the IgG titer. Antibody titer that was higher than mean +3× standard deviation of the control group was considered elevated. Mouse polyclonal anti-*P. gingivalis* HSP antisera that showed elevated IgG responses to *P. gingivalis* HSP were pooled and used for Western immunoblot and opsonophagocytosis assay.

Except for recombinant *P. gingivalis* HSP60, heat-shocked bacterial cells were lysed, respectively, in SDS-PAGE sample buffer. Recombinant *P. gingivalis* HSP60 10 μg, or 100 μg bacterial cell lysates of the target bacteria were loaded to each lane after denaturing at 100°C for 10 min in the presence of 5% β-mercaptoethanol for SDS-PAGE. To each lane, we loaded 100 μg of cell lysate proteins in cases of the other bacteria for semiquantitative comparison of blotting intensity. This was because the heat-inducible protein, representing 1–2% of total bacterial proteins, under normal conditions increases four- to fivefold under heat shock treatment (17). The bacterial proteins were electro-transferred to nitrocellulose membrane. The unoccupied areas on the membrane were blocked by 5% nonfat dry milk. After washing the membrane, anti-*P. gingivalis* HSP antisera was applied. The membrane was washed 5 times with 20 mM Tris buffer (pH 7.5) containing 0.5 M NaCl and 0.1% Tween20 followed by incubation with HRP-conjugated goat anti-mouse IgG. The membrane was extensively washed and bound antibodies were visualized by tetramethylbenzidine. Cell lysates from bacteria grown at 37°C were used for comparison. The cross-reactivity of anti-*P. gingivalis* HSP antisera with human HSP60 (StressGen Biotechnology, Victoria, Canada) was also evaluated.

For the opsonophagocytosis assay, PMNL (2×10^6 cells) isolated from human peripheral blood of healthy subjects without evidence of periodontal or

systemic diseases was incubated with 5 μl of mouse polyclonal anti-*P. gingivalis* HSP antisera. Complement (human baby serum as a complement source, 25 μl) and six target bacteria (2.5×10^7 cells/each) were added, respectively, for further incubation. Finally, luminol (5 mM) was added for chemiluminescence reaction. Time-dependent chemiluminescence intensities (mV) were plotted for comparison. Sham-immunized mouse serum was used as the control. All the assays were repeated three times and the mean peak intensity values were subjected to the Student's *t*-test.

All 10 mice immunized with *P. gingivalis* HSP 60 demonstrated elevated anti-*P. gingivalis* HSP60 IgG antibody titers when compared with sham-immunized control mice. The antisera from each immune mouse also had elevated antibacterial IgG antibody titers against the six putative periodontopathogenic bacteria grown under heat shock when compared with the control mice, but the antisera did not shown an elevated antibody response to the target bacteria grown under normal conditions (Table 1). Mouse anti-*P. gingivalis* HSP60 antisera recognized and reacted with *P. gingivalis* HSP as well as with all other HSPs induced by heat-shock of the putative periodontopathogenic bacteria tested, whereas it did not react with cell lysates from bacteria grown under normal conditions (Fig. 1A). Mouse anti-*P. gingivalis* HSP60 antisera also showed reactivity with homologous *P. gingivalis* HSP 60 and cross-reactivity with human HSP60 (Fig. 1B). Polyclonal mouse anti-*P. gingivalis* HSP60 antisera yielded significantly higher levels of opsonophagocytosis to all six target bacteria than did sham-immunized control sera ($P < 0.01$, Table 2). Time-dependent chemiluminescence intensity curves were higher than those of the control sera (Fig. 2). However, there was no increase in the opsonophagocytosis of the immune antisera against the bacteria grown at 37°C compared with the control serum (Table 2).

We report for the first time that *P. gingivalis* HSP60 could be used as a candidate antigen to cross-protect multiple putative periodontal pathogens as evidenced by the elevated IgG titers to multiple putative bacteria, cross-recognition of heat-induced bacterial proteins by the antisera at a molecular level, and a cross-opsonophagocytosis function of the antisera against the multiple target bacteria. To validate the vaccine efficacy, we have previously reported *in vivo* data that *P. gingivalis* HSP vaccine could reduce the alveolar bone loss induced by multiple periodontopathogenic bacterial infections in the rat (5).

Several candidate antigenic determinants may share the same sequence homology among periodontopathic bacteria. These antigens may include phosphorylcholine (7), capsular polysaccharide (12), and HSP (8). We excluded the use of polysaccharide as a candidate antigen in our study because it has not been identified in *P. gingivalis*. Capsular polysaccharide was not considered either as it could not be a potent inducer of T-cell-mediated immunity, thus requiring capsular polysaccharide–protein conjugation in the vaccine design (2). We have therefore selected HSP, which has been identified in most putative periodontal pathogenic bacteria with a high level of sequence homology. HSPs are produced by all cells in response to various physiological and nonphysiological stimuli (13).

Synthesis of HSP is induced not only by elevated temperature but also by a variety of environmental insults, including changes in pH, or oxygen pressure, confrontation with reactive oxygen metabolites and nutrient deprivation (9, 17). Presumably, therefore, expression of bacterial HSP would be up-regulated in the subgingival area where active periodontal disease progresses, making it a possible molecular target for anti-HSP antiserum. We have successfully demonstrated the opsonophagocytosis function of anti-*P. gingivalis* HSP60 antisera against heat-treated target bacteria, but not against bacteria grown at 37°C.

A major concern, however, if the complete *P. gingivalis* HSP60 were to be used for vaccination (1, 18), is its sequence homology with human tissue components, which might cause a cross-reactive T-cell immune response, leading to the induction of an autoimmune reaction (3, 4). Indeed, our data have demonstrated the cross-reactivity of anti-*P. gingivalis* HSP60 antisera with human HSP60 (Fig. 1B).

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