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Suppressive Effects of DNA Vaccines Encoding Heat Shock Protein on *Helicobacter pylori*-induced Gastritis in Mice

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We investigated the effect of DNA vaccines encoding *H. pylori*-heat shock protein A and B (pcDNA3.1-*hspA* and -*hspB*) on inducing immune responses against *H. pylori* in mice. C57BL/six mice aged 5 weeks were immunized by single injection of 10 μg of pcDNA3.1-*hspA* and pcDNA3.1-*hspB* into intracutaneous tissue. Plasmid DNA lacking the inserted *hsp* were injected as a control. Three months after vaccination, significant specific antibodies against *H. pylori* were detected by ELISA in the sera of vaccinated mice. Antibody isotypes were predominantly IgG2a (Th1-like) with pcDNA3.1-*hspA* and mixed IgG1/IgG2a (Th0-like) with pcDNA3.1-*hspB*. DNA vaccination dramatically suppressed colonies of bacteria in stomach of vaccinated mice (28,400 ± 21,600/mm^2 for pcDNA3.1-*hspA* and 6800 ± 3470/mm^2 for pcDNA3.1-*hspB*) compared to control mice (128,000 ± 42,200/mm^2). Histological analysis of the gastric mucosa demonstrated that the degree of gastritis was significantly lower in the vaccinated mice than in control mice. These results demonstrated that DNA vaccines encoding *H. pylori*-Hsp induce significant immune response against *H. pylori* to decrease gastric mucosal inflammation, indicating that a DNA vaccine can be a new approach against *H. pylori* in humans.

H. pylori is a spiral-shaped microaerophilic bacterium which colonizes the gastric mucosa of humans. It is the principal cause of chronic active gastritis (1), peptic ulcer (2), and categolized as a class I carcinogen for gastric cancer (3). After infection, it is difficult for a host to eliminate the bacteria from the gastric mucosa in spite of specific immune responses to *H. pylori*. Currently, standard treatment for *H. pylori* infection consists of antibiotics together with a proton pump inhibitor, which results in eradication the bacteria in more than 90% of cases (4).

Because it is feared that antibiotic-resistant strains will emerge (5), there is urgent need to find other approaches for the treatment and prevention of this universal infection. The most attractive new strategy would be the development of an effective vaccine against *H. pylori*. In recent years, administration of plasmid DNA (DNA vaccine) was demonstrated to induce both humoral and cellular immunity, and it has become a promising approach against viral, parasitic, and bacterial pathogens against a variety of animal species. In animal models of human disease, protective responses against HIV (6), herpes simplex virus (7, 8, 9), influenza virus (10, 11), rabies virus (12), malaria (13), leishmaniasis (14), and tuberculosis (15, 16) have been induced by DNA vaccination. To our knowledge, a study of a DNA vaccine against *H. pylori* has not been reported so far. In this study, the protective efficacy of a DNA vaccine against *H. pylori* using HspA and HspB genes was evaluated.

We demonstrate that immunization with plasmid DNA encoding HspA or HspB antigens elicite protective immune responses in a mouse model.

MATERIALS AND METHODS

Bacteria and culture conditions. A mouse adapted *H. pylori*, Sydney strain 1 (SS1) (17) was kindly provided by Professor Adrian Lee (School of Microbiology and Immunology, University of New South Wales, Australia), and was used in this study.

Bacteria were cultured at 37°C in Brucella broth (BBL, Cockeysville, USA) containing 3% FBS (Gibco-BRL, UK) and Skirrow's supplement (SR069E; Oxoid, Basingstoke, UK) under microaerobic conditions with gas generator envelopes (Campy Pak Plus and Gas Pak; BBL, Cockeysville, USA) in gas jars. *E. coli* JM109 which was

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used for all cloning experiments was grown at 37°C in LB medium containing 50 mg of ampicillin per liter.

Preparation of H. pylori-DNA for DNA vaccine. *H. pylori* genomic DNA was prepared as described previously (18). Briefly, bacteria were collected by centrifugation for 2 min at 12,000*g*, resuspended in 400 μl of 10 mM Tris–HCl (pH 8.0) containing 5 mM EDTA, 0.1% sodium dodecyl sulfate and 0.1 mg/ml proteinase K, and incubated for 1 h at 37°C before being treated for 10 min at 65°C with CTAB (10% hexadecyletrimethylammonium bromide in 0.7 M NaCl) and 5 M NaCl. The DNA was phenolized and precipitated with ethanol. After washing twice with 70% ethanol, the DNA was dissolved in 100 μl H_2O, and used directly as a template for the polymerase chain reaction (PCR). The primers for *hspA* and *hspB* were designed according to a previous report (19). The HspA gene was amplified using the forward primer; *hspA*-F (5′ ATTATTGAATTCAATCACAAAAAACACTAGTAC 3′) containing an Asp718 site, and the reverse primer; *hspA*-R (5′ ATTCCTATGGTACCCCGTTTTCTTTAGTTTTAAA 3′) containing an *Eco*RI site. The HspB gene was amplified using the forward primer; *hspB*-F (5′ TTCGTTGAATTCAATGTAGTACGGCGGGTACGG 3′) containing an Asp718 site and the reverse primer; *hspB*-R (5′ TTTAGGGTACCCTGTTGCGGGAGGAAAAGATTA 3′) containing an *Eco*RI site. The PCR mixture (50 μl) consisted of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 4.0 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 1.0 U of AmpliTaqGold DNA polymerase (Perkin-Elmer, Norwalk, Conn.), 20 pmol of each primer, and 1 μl of *H. pylori* genomic DNA. PCRs were comprised of 40 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C. Prior to cycling, the samples were heated at 95°C for 15 min to activate the Taq DNA polymerase. The PCR products were separated using QIAquick Gel Extraction Kit (QIAGEN, CA). The purified *hspA* and *hspB* DNA fragments were each subcloned into TA cloning vector pGEM-T (Promega, Madison, WI) having the sequence of β-galactosidase in the multicloning site following the manufacturer's recommendations (pGEM-*hspA* and pGEM-*hspB*). These plasmids were identified their nucleotide sequences using an Applied Biosystems model automatic sequencer.

Plasmid construction for DNA vaccine. The fragments of Asp718 and *Eco*RI-digested pGEM-*hspA* or pGEM-*hspB* were inserted into the Asp718/*Eco*RI site of pcDNA 3.1 (Invitrogen, San Diego, CA) (pcDNA 3.1-*hspA* and pcDNA 3.1-*hspB*). pcDNA3.1 containing no insert was used for as a control (control DNA). The *hspA* and *hspB* DNA sequences in these fainal plasmids were confirmed again.

Immunization and challenge. Female 5-week-old C57/BL6 mice were used for this study. For immunization, mice were injected intracutaneously with 0.1 ml of saline containing 10 μg of pcDNA 3.1-*hspA*, pcDNA 3.1-*hspB*. Three months after immunization, mice were given one orogastric dose of 10^8 *H. pylori* SS1.

Sample collection. Blood was taken from an orbital vein of each mouse 3 months after immunization, and was used to measure anti *H. pylori*-specific antibodies. Six months after the challenge, the animals were killed and assessed for *H. pylori* infection and gastritis. A 5 mm square piece of the antral portion of each stomach was homogenized in phosphate-buffered saline (PBS), and spread on the *H. pylori* selective agar plates (Eiken Chemical Co., Tokyo, Japan). After 5 days of incubation, bacteria were counted as colony forming units (CFU). The remainder of the specimens were used for grading *H. pylori* colonization and mucosal inflammation by histologic examination.

Histological examination. Each stomach section was embedded in OCT (Tissue-Tek; Sakura Finetech, Tokyo, Japan), snap frozen in liquid nitrogen, and stored at −70°C. Cryosections cut at 5 μm were dried overnight at room temperature, post-fixed in acetone for 10 min, and stained with heamatoxylin and eosin. The degree of antral gastritis was scored using a scale of 0 to 3 modified from that of Marshall and Warren (20) i.e.; 0, intact mucosal lining and essentially no infiltration of the lamina propria with monocytes; 1, mild increase in mononuclear infiltration, localized in the upper half of the mucosa; 2, mononuclear infiltration extending from the surface into the lamina propria resulting in atrophy; 3, marked mononuclear infiltration extending from the surface into the lamina propria and disrupting the structure of the glands and leading to marked atrophy, and/or polymorphonuclear leukocyte infiltration in glands and surface erosions. Each stained specimen was examined blindly by a pathologist. To determine the presence of *H. pylori* on gastric mucosa.

The diaminobenzidine (DAB) peroxidase immunohistochemistry technique was used. In brief, sections fixed in acetone were washed in PBS at 4°C and immunolabeled with rabbit antiserum to *H. pylori* (1:100 dillution; DAKO) for 1 h. Antimouse immunoglobulin-horseradish peroxidase conjugate (1:200 dillution; Histofine simple stain PO; NICHIREI, Tokyo, Japan) was then applied for 30 min. Unbound antibody was removed by washing in PBS and then each section was incubated for 4 min in medium containing 50 mM Tris–HCl buffer (pH 7.6), 0.02% (wt/vol) DAB and 0.01% hydrogen peroxide, washed well in distilled water, counterstained with hematoxylin, air dried, and mounted.

Quantitation of the anti-H. pylori antibody response. An enzyme-linked immunosorbent assay (ELISA) was used to detect anti-*H. pylori* specific IgG and IgA antibody in the sera of mice immunized with DNA vaccine. Flatt bottom 96-well plates (PVC Microtiter; Dynex Technologies, Inc., USA) were coated with 5.0×10^4 *H. pylori* in PBS per well. *H. pylori* antigen were fixed on the bottoms of the wells by centrifugation for 10 min at 100*g*. The plates were washed three times in PBS and then blocked with PBS containing 0.02% NaN_3, 0.1% BSA and 1.0 mg of gelatin per ml over night at room temperature. The plates were then washed three times before the addition of each dilluted sample and then incubated at room temprature for 2 h. The plates were were then washed five times with PBS and then reacted for 2 h with a 1:8000 dilution of horseradish peroxidase labeled goat anti-mouse IgG, IgG1, and IgG2a antibodies (Southern Biotechnology, Birmingham, AL) in Tris saline, pH 7.5 containing 0.1% BSA, washed five times in PBS and developed for 15 min with 3, 3′, 5, 5′-tetramethylbenzicline dehydrochloride (BM blue POD substrate precipitating; Boehringer, Mannheim, Germany). The reaction was stopped by addition of 1N-H_2SO_4 to each well, and plates were read at 450 nm in an automatic microplate reader. The endpoint was defined as the highest dilution of serum giving an O.D. at 450 nm greater than 2 S.D. obtained using nonimmune sera.

Statistical analysis. Data are presented as the arithmetic means ± standard errors (S.E.M.). Statistical differences among groups were identified using one-way analysis of varience and multiple comparisons were performed using the least significant difference method. Differences were analyzed by the Student's *t*-test between two groups with statistical significance set at the 5% confidence interval.

RESULTS

Circulating antibodies. Serum anti-*H. pylori* IgG and IgA antibody responses to DNA immunization were shown in Fig. 1. Immunization with pcDNA 3.1-*hspA* or with pcDNA 3.1-*hspB* significantly induced IgG antibody responses in mice compared to immunization with control DNA without inserts. There were no significant differences in the IgG antibody responses between immunization with pcDNA 3.1-*hspA* or pcDNA 3.1-*hspB* (Fig. 1A). Both pcDNA 3.1-*hspA* and pcDNA 3.1-*hspB* also induced anti *H. pylori* IgA antibody responses in mice while vaccination with control DNA showed no significant IgA response. Mice vaccinated with pcDNA 3.1-*hspA* showed more signif-

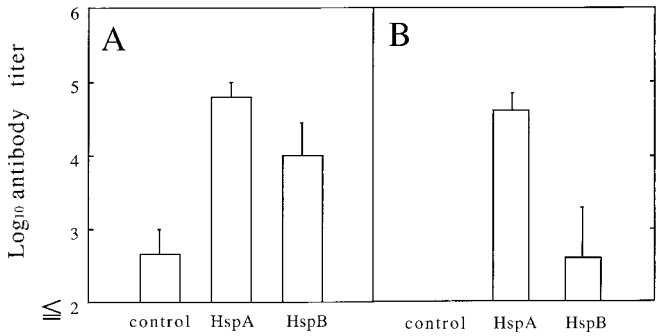

FIG. 1. Serum *H. pylori*-specific IgG (A) and IgA (B) antibody responses (means ± S.E.M.) to pcDNA3.1 (control; $n = 5$), pcDNA3.1-*hspA* (HspA; $n = 5$), and pcDNA3.1-*hspB* (HspB; $n = 5$) administreted intracutaneously.

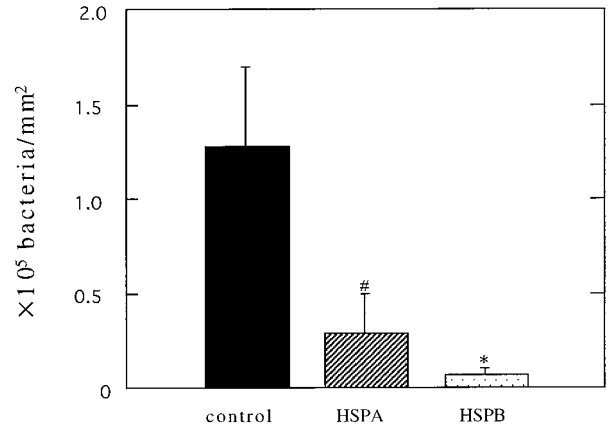

FIG. 2. Protection against *H. pylori* by DNA vaccine. Mice were immunized with pcDNA3.1 (control; $n = 6$), pcDNA3.1-*hspA* (HspA; $n = 4$) to pcDNA3.1-*hspB* (HspB; $n = 5$) by subcutaneous injection. After 3 months, they were challenged with *H. pylori* SS1 (10^8 bacteria). Each stomach was collected 6 months later and dissociated. Their bacterial loads were then evaluated after appropriate dilutions and growth on agar medium. Data are represented as the mean ± S.E.M.; * = $P < 0.01$, # = $P = 0.078$.

icant immune responses in IgA than those vaccinated with pcDNA 3.1-*hspB* (Fig. 1B). To assess the nature of the immune responses to the each plasmid, immunoglobulin isotype profiles were investigated (Table 1). Sera from pcDNA 3.1-*hspA* injected mice contained higher IgG2a antibody titers compaired to IgG1 (Th1-like response), while sera from pcDNA 3.1-*hspB* injected mice had no dominant isotype (Th0-like response).

Quantative analysis of H. pylori colonization. Bacterial CFUs in stomach measured after the DNA vaccines were shown in Fig. 2. Vaccinations conferred significant protection against replication of *H. pylori* in stomachs of B57BL/6 mice following a subsequent challenge but protection with pcDNA3.1-*hspB* (5.3%) was stronger than with pcDNA3.1-*hspA* (22.2%).

Histological findings and analysis of gastritis score. Typical histological findings of gastric mucosa for control and immunized mice with pcDNA 3.1-*hsp* were shown in Fig. 3. In control mice, a lot of inflammatory cell infiltration (Fig. 3A) were observed on the surface and in the gastric mucosa. In vacctinated mice however, much less or very few inflammatory cell infiltration (Fig. 3B) were dettected. Gastric inflammation were semi-quantativly scored and illustrated in Fig. 4. The inflammation scores were significantly lower in both pcDNA 3.1-*hspA* (46.5% reduction) and pcDNA 3.1-*hspB* (16.5% reduction) immunized mice groups than in control group (100%). Colonization with *H. pylori* was verified histologically. There were a lot of bacteria colonies in the mucous layer of stomachs in all control group mice and fewer in HspA and HspB group mice. The values measured by two different methods, CFU and microscopy, almost agreed.

TABLE 1

The Ratio of IgG2a/IgG1 Antibodies

Vaccine	IgG2a/IgG1
HspA	5.3
HspB	1.4

Note. Pooled sera from five mice were analyzed 3 months after immunization with pcDNA3.1-*hspA* (HspA) and pcDNA3.1-*hspB* (HspB) DNA vaccines.

DISCUSSION

It has been shown that immunization with DNA vaccines encoding viral or bacterial antigens can elicit both humoral and cellular immune responses in rodents and nonhuman primates (21, 22, 23). DNA vaccine is easy to produce and purify, and can be stored for a long time because of its biological stability. It is useful for the development of various vaccines and will likely be practical for use in humans in the near future. HspB has been detected on the surface of *H. pylori* in human gastric biopsies (24), and has been previously reported to be a protective antigen in a conventional vaccine (25). HspA may have a role in the interaction between HspB and urease in the chelation of nickel ions, and may be a candidate antigen for a vaccine against *H. pylori* (26).

This study demonstrated humoral responses and the protective ability of DNA vaccines encoding *H. pylori*-HspA or B using the plasmid pcDNA3.1. This plasmid contains the neomycin resistance marker which contains CpG motifs that have been reported to be immunostimulatory (27). The type of immune response reportedly differs with different routes of inoculation such as intramuscular, intravenous, intranasal, and intracutaneous. Intracutaneous injection of plasmid DNA efficiently and reliably primes

humoral immune responses (28, 29). Since humoral immune responses are considered to play an important role in protection against *H. pylori* in stomach (30, 31, 32), we selected the intracutaneous route for immunization and investigated the humoral response and protective efficacy after vaccination with plasmids encoding HspA or HspB. Humoral immune responses were examined in mice vaccinated with either pcDNA3.1-*hspA* or pcDNA3.1-*hspB*. The *hspA* immunized mice acquired a stronger response with both IgG and IgA than the hspB immunized mice (Fig. 1). However, the protective response in the *hspA* immunized group was not significantly stronger compared to that in the *hspB* immunized group (Fig. 2). The IgG antibody isotypes were different in the *hspA* and *hspB* immunized groups, indicating that the *hspA* immunized mice acquired Th1-like immune responses, and that *hspB* immunized mice acquired Th0-like immune responses (Table 1). This

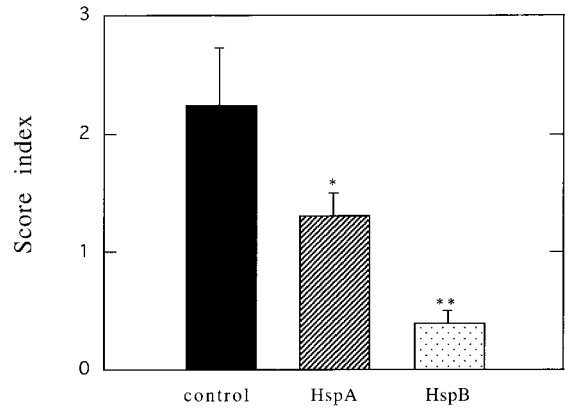

FIG. 4. Effect of DNA vaccination with pcDNA 3.1 (control; $n = 5$) pcDNA3.1-*hspA* (HspA; $n = 4$), and pcDNA3.1-*hspB* (HspB; $n = 5$) on gastritis. Data are represented as the means ± S.E.M. * = $P < 0.05$ and ** = $P < 0.01$.

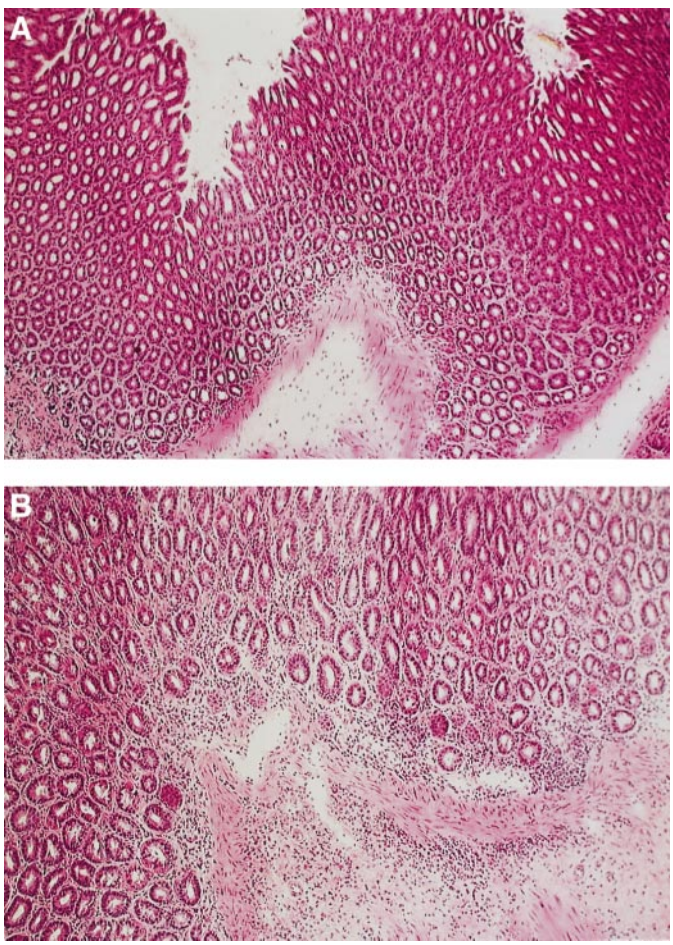

FIG. 3. (A) Animal vaccinated with pcDNA3.1-*hsB*. There are normal epithelial cells both on the surface and within the glands and only minimal mononuclear infiltration (gastritis grade of 0.5). (B) Animal vaccinated with pcDNA3.1 (control). There is intense mononuclear infiltration especially on the surface but also through the lamina propria (gastritis grade of 3.0).

difference probably led to the result that the protective activity in the *hspA* immunized mice was less than in the *hspB* immunized mice. Considering the immune responses, protective efficacies, and gastritis scores, pcDNA3.1-*hspB* seemed to be a better vaccine than pcDNA3.1-*hspA*. DNA vaccination with pcDNA-*hspB* supressed colonization of *H. pylori* strongly, but did not protect against the infection perfectly. To protect against *H. pylori* infection perfectly, it may be necessary to select more effective DNA antigens and use combination of different plasmid DNA antigens along with adjuvant which have the potential for improving the immune response (33, 34). Because decreasing *H. pylori* in the stomach led to the reduction of gastritis grade in this mouse model, adequate supression without perfect protection against *H. pylori* probably can prevent intense pathogenesis. Therefore, perfect protection against *H. pylori* may not be necessary. We plan to investigate the efficacy of DNA vaccines in the pathogenesis of gastritis, peptic ulcer, and gastric carcinoma using the Mongolian gerbil (35, 36) which is an excellent model for studying *H. pylori* infection, and its relationship to its pathogenesis.

In summary, our results suggest that a DNA vaccine can give significant protection against an orogastric *H. pylori* challenge. The DNA vaccine strategy presented here is easy and should be a promising method for identifying antigens which might be capable of conferring protection against *H. pylori* infection.

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