

**EFFECTIVENESS OF INTRAGASTRIC IMMUNIZATION WITH PROTEIN
AND OLIGODEOXYNUCLEOTIDES CONTAINING A CpG MOTIF
FOR INDUCING A GASTROINTESTINAL MUCOSAL
IMMUNE RESPONSE IN MICE**

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Abstract : [Purpose] To investigate a new modality of mucosal vaccines, we evaluated the effectiveness of intragastric immunization for inducing a mucosal immune response in the gastrointestinal tract. [Methods] Mice were immunized with β -galactosidase (β -gal) and synthesized oligodeoxynucleotides containing a CpG motif (CpG-DNA) by intragastric injection, and the immune response was compared with those induced by 3 other immunization forms: intranasal, oral, and intradermal. [Results] Intragastric immunization with β -gal and CpG-DNA induced significant anti- β -gal fecal IgA production at 2 weeks; however, at 4 weeks the response was lacking. In contrast, intranasal immunization with β -gal and CpG-DNA induced the highest anti- β -gal fecal IgA production at 4 weeks. [Conclusion] Although intragastric immunization with protein and CpG-DNA induces a mucosal immune response in the gastrointestinal tract, intranasal immunization is the most effective to induce both mucosal and systemic immune responses. This finding may increase the possibility for developing vaccines against mucosal pathogens, especially *Helicobacter pylori*.

Key words : mucosal vaccine, mucosal immune response, intragastric immunization, CpG motif, *Helicobacter pylori*

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INTRODUCTION

Vaccination is a potent modality of preventing infectious diseases. However, new and better vaccines are always pursued because there are serious deficiencies in traditional vaccines. Furthermore, new and emerging pathogens represent major health threats. Recently, there is a great interest in developing mucosal vaccines. The gastrointestinal (GI), respiratory, and vaginal mucosa are major portals of entry for pathogens, and the mucosal immune system serves as a front line of defense in protection from infection. Traditional protein-based vaccines have not only no effect in preventing the entry of pathogens at the mucosal site, but also have the possibility of inducing tolerance if delivered orally. Therefore, developing a new vaccine that induces mucosal as well as systemic immune responses without inducing tolerance is one of the major goals in mucosal vaccine design.

In a previous study, we successfully transfected plasmid DNA into gastric mucosal epithelial cells by intragastric (i.g.) injection¹⁾. This technique induced not only IgA production in the GI tract, respiratory tract, and vagina, but also major histocompatibility complex (MHC) class I-restricted cytotoxic T-lymphocyte (CTL) in local lymph nodes and spleen. These findings demonstrated that gastric mucosa is a part of the common mucosal immune system, and that i.g. immunization with plasmid DNA induces antigen-specific humoral and cellular immune responses mucosally as well as systemically, without inducing tolerance. Therefore, we proposed the application of this method to GI infection, particularly to *Helicobacter pylori* (*H. pylori*) infection.

Gene vaccination with plasmid DNA induces long-lived and potent immune responses. For more than a decade, gene vaccination has been gaining attention as a potential approach to the development of vaccines against infectious diseases. Because application of plasmid DNA provides protection against various pathogens in experimental animal models, clinical trials of many different DNA vaccines have been performed or are in progress for various diseases²⁾. One of the proposed mechanisms of how plasmid DNA induces immune responses is that microbial DNA, containing unmethylated CpG motifs in the plasmid backbone, provides a strong adjuvant effect in stimulating the innate immune system. The CpG motif can act as an adjuvant when it is in the plasmid backbone or in synthesized oligodeoxynucleotides. Recent studies have shown that mucosal immunization with protein and synthesized oligodeoxynucleotides containing the CpG motif (CpG-DNA) induces mucosal immune responses with a wide range of antigens. Several forms of mucosal immunization are available in experimental animal models, especially, intranasal immunization is considered to be useful in inducing both mucosal and systemic immune responses³⁾. However, no study has tested immunization with a mixture of protein and CpG-DNA by intragastric injection.

In this study, we immunized mice intragastrically with protein and CpG-DNA,

instead of plasmid DNA, to investigate the new modality of mucosal immunization and to identify the optimal form of immunization in inducing the most effective immune response in the GI tract.

METHODS

Animals

This study was carried out under the control of Animal Experiments in Fukushima Medical University and Japanese Government Animal Protection and Management Law (No. 105). Female BALB/c mice, aged 8-12 weeks, were purchased from Charles River Japan (Yokohama, Japan) and used in all experiments.

Reagents

β -Galactosidase (β -gal) (Sigma, MO, USA) and synthesized CpG-DNA (Bex, Tokyo, Japan) were used for immunization. The CpG-DNA had the following sequence: 5'-TCCATGACGTTTCCTGATGCT-3'.

Immunization protocols

Thirty mice were divided into 6 groups of 5 each, and each group was assigned to one of the following forms of immunization: i.g. immunization with saline (i.g. saline), i.g. immunization with 10 μ g of β -gal (i.g. β -gal), i.g. immunization with 10 μ g of β -gal and 40 μ g of CpG-DNA (i.g. β -gal/CpG-DNA), intranasal (i.n.) immunization with 10 μ g of β -gal and 40 μ g of CpG-DNA (i.n. β -gal/CpG-DNA), oral (p.o.) immunization with 10 μ g of β -gal and 40 μ g of CpG-DNA (p.o. β -gal/CpG-DNA), and intradermal (i.d.) immunization with 10 μ g of β -gal and 40 μ g of CpG-DNA (i.d. β -gal/CpG-DNA).

For i.g. immunization, mice were anesthetized peritoneally with 1.25 mg of pentobarbital sodium (Dainabot, Osaka, Japan), and a small incision was made with a scalpel at the upper middle portion of the abdomen. The stomach was exposed, and 25 μ l of solution was injected into the anterior gastric wall with a 27-gauge needle. The incision was immediately closed after immunization. For i.n. immunization, mice were anesthetized, and 15 μ l of the solution was delivered to each naris. For p.o. immunization, mice were fasted for 5 hours before immunization, and 200 μ l of solution was delivered through polyethylene tubes (CLEA Japan, Tokyo, Japan) inserted into the mice orally. For i.d. immunization, 50 μ l of solution was injected at the base of the tail with a 27-gauge needle.

Collection of samples

Feces were collected every week after immunization, and fecal extracts were prepared as previously described³⁹. In brief, 3 to 4 pieces of freshly voided feces were collected and their weights were recorded. The feces were resuspended in 2% bovine serum albumin (BSA) in PBS at a ratio of 10 μ l/mg and subjected to

vortexing for 60 minutes. The extracts were spun to remove debris, and the supernatant was collected and stored at -20°C . Mice were bled every week and sera were stored at -20°C . Bronchoalveolar lavage fluids (BALF) were obtained from sacrificed mice by lavage with 1 ml of PBS at 2 and 4 weeks. The return was spun to remove cellular debris, and the supernatant was frozen at -20°C until the IgA assay was performed.

Cell culture

Spleens were harvested from sacrificed mice 4 weeks after immunization for the cytokine assay as previously described⁴¹. Three days before sacrifice, mice were intravenously boosted with 10 μg of β -gal. Mice were sacrificed by CO_2 inhalation, and spleens were harvested and teased to make single cell preparations. Splenocytes were resuspended in RPMI 1640 supplemented with 10% FBS, 2 mM penicillin/streptomycin, and 50 μM β -mercaptoethanol at a concentration of $2 \times 10^6/\text{ml}$. Culture supernatants were harvested after incubation with or without β -gal (10 $\mu\text{g}/\text{ml}$) for 72 hours at 37°C in 5% CO_2 . Levels of IFN- γ and interleukin (IL)-4 in the supernatants were analyzed by enzyme-linked immunosorbent assays (ELISA).

Antibody assays

Fecal extraction fluid, serum, and BALF were used in ELISA for the antigen-specific immunoglobulin assay as previously described³¹. Results were expressed in units/ml based on pooled high-titer anti- β -gal standards obtained from immunized mice. Ninety-six-well plates were coated with 5 $\mu\text{g}/\text{ml}$ of β -gal in PBS overnight at 4°C . After incubation with 2% BSA/PBS for 2 hours at room temperature, plates were washed with 0.1% Tween 20 in PBS and incubated with samples and standards overnight at 4°C . Plates were washed and incubated with alkaline-phosphatase-linked anti-IgA or anti-IgG (Southern Biotechnology Associates, AL, USA) for 2 hours at room temperature. After washing, plates were incubated with a solution of *p*-nitrophenyl phosphate (1 mg/ml). Absorbance at 405 nm was read and compared to the standard curve on each plate using Microplate Reader and Microplate Manager ver. 5.1 Program (Bio-Rad, CA, USA).

Cytokine assay

Purified rat anti-mouse IFN- γ and IL-4 capture antibodies (BD PharMingen, CA, USA) were diluted and added to the ELISA plates. After incubation overnight at 4°C , the capture antibody solution was removed and plates were incubated with 2% BSA/PBS for 2 hours at room temperature. After washing, plates were incubated with samples and standards overnight at 4°C . After washing more than 5 times, biotinylated anti-mouse IFN- γ and IL-4 detection antibodies (BD PharMingen, CA, USA) were added, and plates were incubated for 1 hour at room temperature. After washing, Avidin-Horseradish Peroxidase (Zymed, CA, USA), diluted in 2% BSA/PBS, was added, and plates were incubated for 1 hour at room tempera-

ture. After washing, TMB solution (DAKO, CA, USA) was added for color development. The color reaction was stopped by adding 1 M phosphoric acid. Absorbances were read at 450 nm and the results were expressed in pg/ml using Microplate Reader (Bio-Rad, CA, USA).

Statistics

The antibody titers and the cytokine levels were expressed as the mean \pm standard error. The data were analyzed for statistical significance by unpaired two group t-test or ANOVA using StatView J-5.0 computer software (SAS Institute Inc., NC, USA). Fisher's exact probability test was used to establish *P* values, and those <0.05 were considered significant.

RESULTS

Intragastric immunization with β -gal and CpG-DNA elicits antigen-specific IgA production in the GI tract, but intranasal immunization induces longer and stronger responses

Intragastric β -gal/CpG-DNA elicited the production of anti- β -gal IgA in the GI tract, although the titer did not rise as high as that when i.n. β -gal/CpG-DNA did (Fig. 1A). At 2 weeks, both i.g. and i.n. β -gal/CpG-DNA elicited significantly higher anti- β -gal fecal IgA production (17.5 ± 2.6 and 17.4 ± 3.2 U/ml, respectively) compared to that of i.g. saline (8.1 ± 2.2 U/ml) (Fig. 1B, $P=0.0296$ and $P=0.0444$, respectively). At 4 weeks, however, i.g. β -gal/CpG-DNA did not elicit significant fecal IgA production (16.7 ± 4.4 U/ml), whereas i.n. β -gal/CpG-DNA remarkably did (39.4 ± 7.9 U/ml) compared to that of the other groups (Fig. 1C, $P<0.0001$). The fecal IgA : IgG ratio did not correlate with the serum IgA : IgG ratio (data not shown). These findings demonstrate that although i.g. β -gal/CpG-DNA induces a mucosal immune response in the GI tract, i.n. β -gal/CpG-DNA induces longer and stronger responses.

Intranasal immunization with β -gal and CpG-DNA elicits the highest IgA production in the respiratory tract as well as in the GI tract

In the previous study, i.g. immunization with plasmid DNA elicited significant antigen-specific IgA production not only in the GI tract, but also in the respiratory tract¹¹. In this study, at 2 weeks, i.g. β -gal/CpG-DNA elicited no significant anti- β -gal IgA production in the respiratory tract, but i.n. β -gal/CpG-DNA did elicit significant production (12.7 ± 5.8 U/ml) compared to that of the other groups (Fig. 2A, $P<0.01$). At 4 weeks, i.n. β -gal/CpG-DNA elicited an increased amount of BALF anti- β -gal IgA (33.7 ± 6.4 U/ml), and the titer was significantly the highest compared to that of the other groups (Fig. 2B, $P<0.0001$). Thus, i.n. β -gal/CpG-DNA induced the highest IgA production in the respiratory tract as well as in the GI tract, whereas i.g. β -gal/CpG-DNA did not.

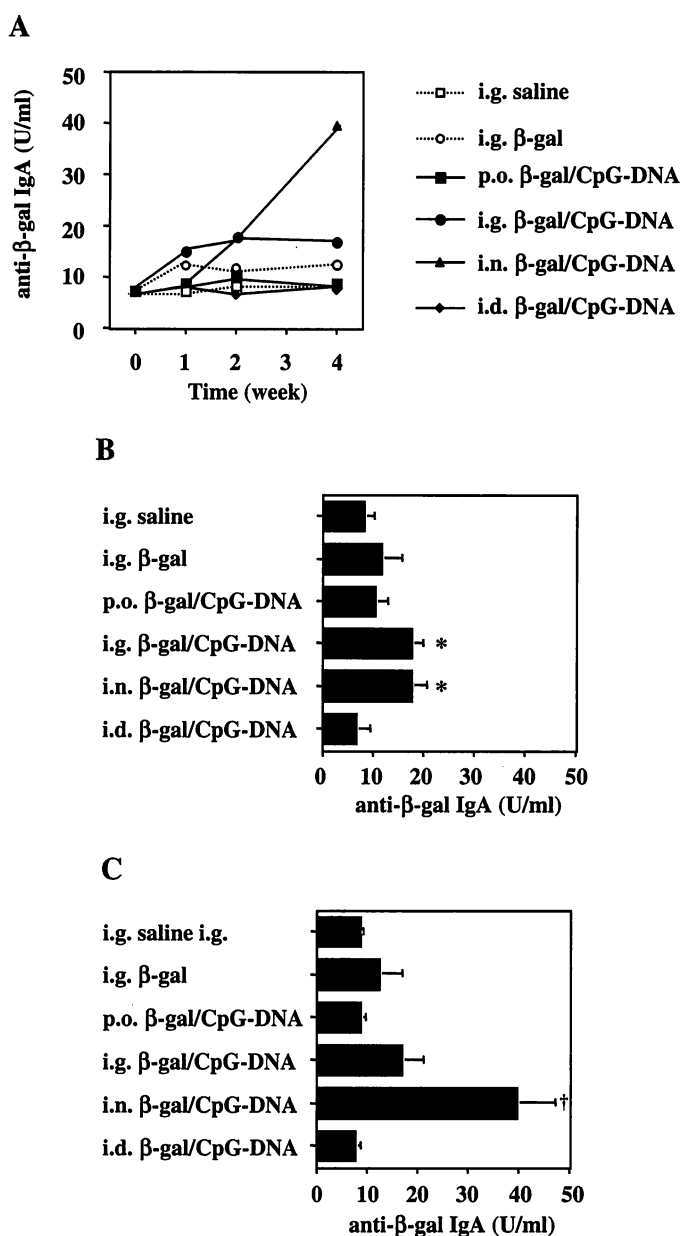


Fig. 1. Fecal IgA responses. Results represent mean values for 5 mice per group, and error bars reflect the standard error of the mean. Results are representative of 3 similar and independent experiments. (A) I.g. β -gal/CpG-DNA and i.n. β -gal/CpG-DNA elicited the production of fecal anti- β -gal IgA. (B) At 2 weeks, both i.g. and i.n. β -gal/CpG-DNA elicited significantly higher anti- β -gal fecal IgA production (17.5 ± 2.6 and 17.4 ± 3.2 U/ml, respectively) compared to that of i.g. saline (8.1 ± 2.2 U/ml) (* $P < 0.05$). (C) At 4 weeks, i.g. β -gal/CpG-DNA did not elicit significant fecal IgA production (16.7 ± 4.4 U/ml), whereas i.n. β -gal/CpG-DNA remarkably did compared to that of the other groups (39.4 ± 7.9 U/ml) († $P < 0.0001$).

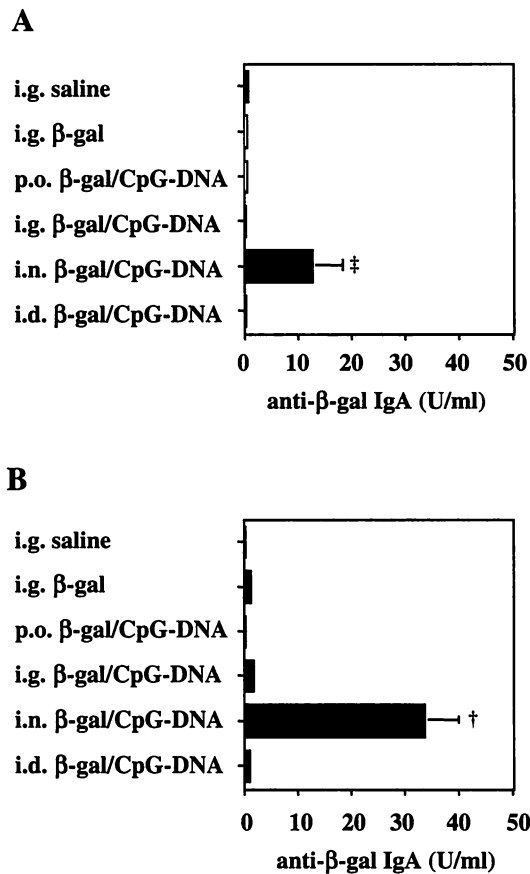


Fig. 2. BALF IgA responses. Results represent mean values for 5 mice per group, and error bars reflect the standard error of the mean. Results are representative of 3 similar and independent experiments. (A) At 2 weeks, i.g. β -gal/CpG-DNA elicited no significant anti- β -gal IgA production in the respiratory tract, but i.n. β -gal/CpG-DNA did (12.7 ± 5.8 U/ml) compared to the other groups ($^{\dagger}P < 0.01$). (B) At 4 weeks, i.n. β -gal/CpG-DNA elicited increased amount of BALF anti- β -gal IgA (33.7 ± 6.4 U/ml), and the titer was significantly the highest compared to that of the other groups ($^{\dagger}P < 0.001$).

Intranasal immunization with β -gal and CpG-DNA elicits the highest serum antibody production

In the previous study, i.g. immunization with plasmid DNA elicited prolonged antigen-specific immunoglobulin production in serum¹¹. In this study, both i.g. and i.n. β -gal/CpG-DNA elicited anti- β -gal IgA production in serum. At 4 weeks, i.n. β -gal/CpG-DNA significantly induced the highest anti- β -gal IgA production (768.8 ± 101.2 U/ml) compared to that of the other groups (Fig. 3A, $P < 0.0001$). Furthermore, i.n. β -gal/CpG-DNA elicited the most significant anti- β -gal IgG production ($5,046.0 \pm 306.0$ U/ml), almost 5-fold higher than did i.d. β -gal/CpG-DNA ($1,037.1 \pm$

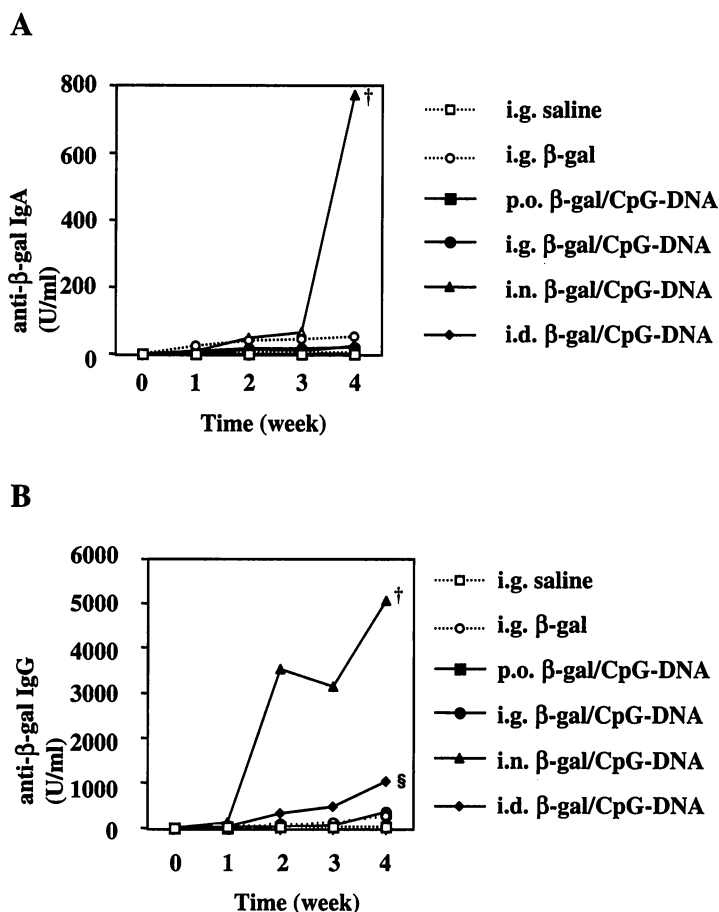


Fig. 3. Serum IgA and IgG responses. Results represent mean values for 5 mice per group. Results are representative of 3 similar and independent experiments. (A) At 4 weeks, i.n. β -gal/CpG-DNA significantly induced the highest anti- β -gal IgA production (768.8 ± 101.2 U/ml) compared to that of the other groups ($^{\dagger}P < 0.0001$). (B) I.n. β -gal/CpG-DNA elicited the most significant anti- β -gal IgG production ($5,046.0 \pm 306.0$ U/ml) compared to the other groups ($^{\dagger}P < 0.0001$), almost 5-fold higher than did i.d. β -gal/CpG-DNA ($1,037.1 \pm 455.4$ U/ml) ($^{\S}P < 0.005$ vs i.g. saline).

455.4 U/ml) (Fig. 3B, $P < 0.0001$). These data demonstrate that i.n. β -gal/CpG-DNA induces the highest antigen-specific IgA and IgG production in serum.

Intranasal immunization with β -gal and CpG-DNA elicits the best Th1-biased cellular response

To investigate T helper cell differentiation induced by various forms of immunization with β -gal/CpG-DNA, IFN- γ and IL-4 production was evaluated from splenocytes pulsed with β -gal *in vitro*. Four weeks after immunization, i.n. β -gal/CpG-DNA significantly elicited the highest IFN- γ production ($1,225 \pm 352$ pg/ml)

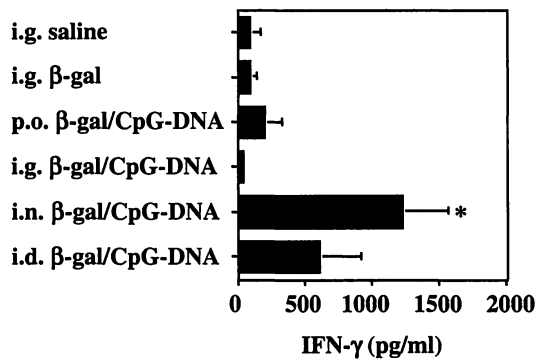


Fig. 4. IFN- γ production from splenocytes. Results are representative mean values for 5 mice per group, and error bars reflect the standard error. Results are representative of 3 similar and independent experiments. I.n. β -gal/CpG-DNA significantly elicited the highest IFN- γ production ($1,225 \pm 352$ pg/ml) compared to that of the other groups ($*P < 0.05$). There was no detectable IL-4 in the same culture. Splenocytes cultured without β -gal produced no detectable IFN- γ or IL-4.

compared to that of the other groups (Fig. 4, $P < 0.05$). There was no detectable IL-4 in the same culture. Splenocytes cultured without β -gal produced no detectable IFN- γ or IL-4. These findings show that i.n. β -gal/CpG-DNA induces the best Th1-biased cellular immune response compared to the other forms of immunization.

DISCUSSION

It is crucial for vaccine design to identify an optimal adjuvant and immunization form as well as an antigen or candidate immunogen. In experimental models, cholera toxin (CT) and *Escherichia coli* heat-labile toxin (LT) are potent mucosal adjuvants. However, their inherent toxicity and technical problems have kept them from becoming available for use in humans⁵. It is also known that CpG-DNA is a mucosal adjuvant that induces both mucosal and systemic immune responses to co-administered antigens in experimental models^{3,6,7}.

The CpG motif was first discovered in DNA purified from BCG and shown to induce antitumor activity⁸. This molecule activates innate immunity through Toll-like receptor 9, one of the pattern recognition receptors that recognize pathogen-associated molecular patterns⁹. Activation of the innate immunity by the CpG motif particularly results in a production of IL-12 from antigen-presenting cells to foster a Th1-biased immune response in adaptive immunity¹⁰. The Th1 dominant response generated by immunization with antigen and adjuvant containing the CpG motif has been applied to vaccination or immunotherapy for infectious diseases, neoplasms, and allergic diseases¹⁰. The CpG motif can act as an adjuvant when it is contained in a plasmid backbone or synthesized oligodeoxynucleotides^{11,12}. Recent studies have shown that immunization with protein and CpG-DNA at

mucosal sites induces mucosal as well as systemic immune responses in both humoral and cellular immunity to a wide range of antigens^{3,6,7}). However, no study has ever been made of the immune responses induced by i.g. immunization with protein and CpG-DNA. Thus, we decided to test the adjuvanticity of CpG-DNA and to identify an optimal form of immunization for inducing mucosal immune response in the GI tract.

In this study, a single immunization by i.g. injection with a mixture of protein and CpG-DNA induced antigen-specific IgA production in the GI tract and IgG production in serum. The level of fecal IgA induced by i.g. β -gal/CpG-DNA was significantly higher than that induced by i.g. saline at 2 weeks. However, at 4 weeks there was no significant difference between the levels of fecal IgA in the two groups. The immune responses induced by i.d. injection with protein and CpG-DNA are known to last almost as long as those induced by i.d. injection with plasmid DNA. In this study, i.g. immunization with protein and CpG-DNA did not induce as much mucosal or humoral immune responses as did i.g. immunization with plasmid DNA. Although antigen processing at the gastric mucosa has not been fully investigated, some investigators observed that gastric mucosal epithelial cells presenting MHC class II and accessory molecules, such as B7-1 and B7-2, may play an important role as antigen-presenting cells¹³⁻¹⁵). Because plasmid DNA provides a stable and prolonged source of the antigen¹⁶), immunization with plasmid DNA may be more suitable for antigen processing at the gastric mucosa than immunization with protein and CpG-DNA, when immunized by i.g. injection.

Next, we compared the immune responses induced by the 4 different forms of immunization with protein and CpG-DNA to determine the most efficient form in inducing mucosal immune response in the GI tract. The i.n. immunization induced the highest antigen-specific IgA production in the GI tract. Immunization has been accomplished by p.o. administration of antigens derived from *H. pylori* combined with CT or LT in most previous studies, with varied protective and therapeutic effects^{17,18}). Only a few studies demonstrated that the other forms of mucosal immunization, such as i.n. or rectal, successfully induced mucosal and systemic immune responses¹⁹⁻²¹). In this study, i.n. immunization was the most effective in inducing a mucosal response in the GI tract when a mixture of protein and CpG-DNA was administered. This finding may provide new insight into the development of mucosal vaccines against *H. pylori*. Recently, i.n. immunization with sonicated *H. felis* and a combination of CpG-DNA and CT has shown sterile immunity²²). As for safety in vaccination, the toxicity of antigens derived from *H. pylori* and adjuvants, such as CT and LT, cannot be ignored. It is hoped that safer and more effective antigens, such as recombinant protein or peptides, as well as adjuvants, such as CpG-DNA conjugated with those antigens, will be developed^{23,24}).

In this study, i.n. β -gal/CpG-DNA induced the highest systemic IFN- γ production as well as mucosal immune response. It is still unclear to what extent specific antibodies and cell-mediated immune mechanisms contribute to prophylactic protec-

tion against or eradication of pathogens at mucosal sites. Although there are still conflicting views as to whether Th1 type immune response or Th2 achieves protection against *H. pylori* infection²⁵⁻²⁸⁾, since recent observations suggest that both types do²⁹⁾, systemic IFN- γ production induced by i.n. immunization with protein and CpG-DNA may provide protective and therapeutic effects.

Helicobacter pylori infection causes gastritis and that is associated with the development of gastric cancer and malignant lymphoma. Although antibiotic therapy is available for the treatment of *H. pylori* infection, a rise in the microbial resistance cannot be ignored recently. Patients are infected by *H. pylori* mostly in their childhood, therefore, the protective therapy by vaccine is more suitable than antibiotic therapy. Intranasal immunization has been gaining attention as a potential approach for the development of vaccines that prevent and treat infectious diseases in the respiratory tract³⁰⁾. The characterization and the mechanism of the immune cells in the nasal and gastrointestinal mucosa are not fully investigated. The findings demonstrated here show that intranasal immunization has a potential to prevent infectious diseases not only in the respiratory tract but also in the gastrointestinal tract.

In conclusion, we have found 1) that i.g. immunization with protein and CpG-DNA induces a mucosal immune response in the GI tract, and 2) that i.n. immunization with protein and CpG-DNA induces the most effective mucosal and systemic immune responses compared to i.g., p.o., or i.d. immunization. These findings may increase the possibility for vaccine development against mucosal pathogens, especially *H. pylori*.

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