



The mucosal adjuvant activity of the oligodeoxynucleotides containing a non-methylated CpG motif on BCG and diphtheria toxoid

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ABSTRACT

CpG-DNA is currently attracting attention as an effective and safe vaccine adjuvant to prevent from microbial infections. In this report, we examined the effects of oligo B, which is a synthetic CpG-DNA, in mucosal administration of *Bacillus Calmette-Guérin* (BCG) and diphtheria toxoid (DT). Co-administration with oligo B enhanced BCG-induced delayed type hypersensitivity to purified protein derivative (PPD) in guinea pigs. The titers of anti-DT serum IgG, IgA and mucosal IgA antibodies induced by intranasal administration with DT plus oligo B were significantly higher than that with DT alone. In both C57BL/6 and BALB/c mice, intranasal administration of DT with oligo B induced enough level of antibodies to prevent onset of diphtheria. The analysis of antibody subclasses showed that intranasal administration of oligo B induced not only IgG1 but also IgG2a, IgG2c and IgA anti-DT antibodies. In contrast, there was no or little production of the anti-DT serum IgE. Taken together our data suggest that oligo B is a powerful adjuvant in mucosal immunization.

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1. Introduction

Oral and nasal mucosal immunizations have received a great deal of attention in recent years, because many infectious organisms are transmitted through the mucosal surfaces of the gastrointestinal, genitourinary and respiratory tracts. Most vaccines, however, are given parenterally and thus cannot stimulate mucosal immunity, whereas mucosal immunizations stimulate both mucosal and systemic immunities, and antigen-specific mucosal immune responses can be expressed at all mucosal sites [1,2]. In addition, the inoculation procedure is simple, reliable and cheap, and injection needles are unnecessary. However, mucosal immune responses to protein antigen alone have usually been only weakly demonstrated, if at all, and mucosal adjuvants that can sufficiently enhance mucosal antigen-specific IgA antibody response levels are therefore required for immunization. Cholera toxin (CT), *Escherichia coli*-derived heat-labile toxin, its B subunit

and its mutated modified proteins are generally well-known as mucosal adjuvants [3–8], and a number of mucosal adjuvants have been proposed. There are not many current mucosal vaccines currently available for human use, however, for example the oral polio vaccine and a nasal cold-adapted influenza virus vaccine (Flumist®), which are live attenuated vaccines. In addition to these, however, nasal enterotoxin-adjuvant inactivated influenza vaccine was withdrawn after a short time on the market because of a serious adverse reaction, facial paresis [9]. Therefore, further work is needed to develop mucosal adjuvants for human use.

Tokunaga et al. described first that the nucleic acid fraction of the BCG vaccine inhibited the tumor growth of the guinea pig and that this fraction was DNase-sensitive [10–12]. Moreover, Yamamoto et al. reported that immune-stimulatory activity was present in the oligodeoxynucleotides containing a non-methylated CpG motif (CpG-DNA) [13–16].

Recently it has been clarified that bacterial CpG-DNA is the ligand of TLR9. Interactions between CpG-DNA and TLR9 activates dendritic cells, splenocytes, monocytes and macrophages through the toll-like receptor (TLR) signaling pathway to produce Th1 polarizing and inflammatory cytokines such as IFN- γ , IL-1 β , IL-6 and IL-12 [17]. Systemic administration of CpG-DNA *in vivo* was demonstrated to promote NK cell activity [13–16]. Altogether, CpG-DNA induces predominantly Th1 type immune responses. Therefore,

Abbreviations: CpG-DNA, the oligodeoxynucleotides containing non-methylated CpG motif; BCG, *Mycobacterium bovis* Bacillus Calmette-Guérin; DT, diphtheria toxoid.

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CpG DNA is an attractive candidate for the development of new therapies against cancer and allergy [17–20], and it is currently attracting attention as an effective and safe vaccine adjuvant to defend against bacterial or viral infection [16,17,20]. This study was thus designed to investigate the mucosal adjuvanticity of Oligo B, which is a synthetic CpG-DNA, because a comparison of synthetic oligo DNAs revealed that Oligo B had the strongest activity on IFN induction and NK cell activation [16,22,23]. We think that the function of inducing cellular immunity and humoral immunity can be shouldered by more than one vaccine antigen. Therefore, we used two vaccine antigens, namely, DT, which represents vaccines mainly inducing humoral immunity, and BCG, which represents vaccines mainly inducing cellular immunity, when administered alone.

2. Materials and methods

2.1. Animals

Seven-weeks-old female BALB/c or C57BL/6 mice and female guinea pigs of the Hartley strain, weighing approximately 300 g, were purchased from the Japan SLC Co. (Shizuoka, Japan).

2.2. Test material

CpG-DNA (OligoB: 5'-GGG GGG GGG GGG AAC GTT GGG GGG GGG GGG-3' [16], Negative control: methylated Oligo B) was synthesized by TAKARA-BIO, Inc. A recombinant cholera toxin B subunit (rCTB) was used for the positive control as a mucosal adjuvant [24].

2.3. Immunogens

BCG vaccine, Japanese strain, and purified protein derivative (PPD) were obtained from the Japan BCG Laboratory (Tokyo, Japan). Diphtheria toxoid containing 2064 Lf units m1-1 (611 µg protein nitrogen ml⁻¹, purity; 3378 Lf mg⁻¹ protein nitrogen) was provided by the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan).

2.4. Reagents

The following reagents were obtained from commercial manufacturers: fetal calf serum, penicillin, streptomycin and actinomycin D from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.); WST-1 and 1-methoxy PMS from Dojindo Co. (Kumamoto, Japan); and murine TNF-α from Calbiochem-Novabiochem Co. (La Jolla, CA, U.S.A.).

2.5. Immunization of guinea pigs and skin reaction with PPD or DT

Guinea pigs were immunized intranasally with 10⁵–10⁶ CFU of BCG in the presence or absence of CpG-DNA in 100 µl of saline with a micropipette under light ether anesthesia, administered via the nares. The delayed type hypersensitivity (DTH) skin reaction was assayed in guinea pigs. Six weeks after intranasal administration of BCG, the back of the guinea pig was shaved and PPD was administered intradermally (0.05–0.2 µg) in 0.1 ml of saline. The diameter of the induration was measured 24 h after injection.

DT was administered at 20 Lf/100 µl with or without 50 µg of OligoB, on days 0, 14, 21. Six weeks after DTH reaction were performed with DT (0.05–2 µg/0.1 ml) and seven weeks after guinea pigs were bled.

2.6. Preparation and culture of spleen cells

The immunized mice and guinea pigs were humanely killed, their spleens were collected and spleen cells were suspended

in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (FCS-RPMI) at a concentration of 1 × 10⁷ cells/ml. After the cells were cultured as described previously [25], the supernatants were collected for cytokine assays.

Spleen cells were cultured with PPD in 96-well round-bottomed tissue culture plates using [³H]thymidine as previously described [26], to measure their antigen-specific proliferation. The cells were harvested onto glass fiber filters using a cell harvester. [³H]thymidine incorporation into DNA was measured with a scintillation counter.

2.7. Guinea pig TNF-α and IFN bioassay

TNF-α activity in the supernatant was measured using cytotoxic bioassay on L929 cells as previously described [26]. This assay is based upon quantitation of the cytotoxic activity of TNF-α on L929 cells in the presence of actinomycin D [27].

IFN activity in the supernatant was measured using inhibition of CPE activity on the guinea pig fibroblast cell line 104C1 infected with encephalomyocarditis virus [28]. 3 × 10⁴ cells were cultured in a 96-well plate overnight. The cell monolayers formed were incubated overnight with serial 2-fold dilution of IFN-containing supernatant fluids and then infected with EMCV at 0.03 moi for 24 h. Mouse IFNγ was used as positive control of IFN. The amount of biologically active guinea pig IFN was quantified by estimating viable cell numbers colorimetrically by means of a tetrazolium compound, WST-1 and 1-methoxy-PMS. WST-1 color developed until stopped by the addition of sulfuric acid.

2.8. Immunization of mice and sample collection

Mice were immunized intranasally under light ether anaesthesia with 20 µl of solution containing DT (5 Lf/mouse) and various amount of OligoB (0–200 µg/mouse, on days 0, 14, 21 and 28 and sacrificed on day 35).

Lung and nasal cavity lavages, small and large intestinal washes, saliva and vaginal secretions and blood were collected as previously described [5–7]. Fecal pellets were collected from each immunized mouse and solution II was added to them at a ratio of 10 µl/mg, homogenized and then centrifuged at 16,000 × g for 60 min at 4 °C. The supernatant fluids were stored at –20 °C until use for an ELISA.

Mouse spleen cells were cultured with DT (10 µg/well) in 24-well tissue culture plates as previously described [25]. The concentrations of mouse cytokines in the supernatants were determined with a commercial ELISA kit (Biosource International, Inc., Camarillo, CA, U.S.A.).

2.9. Measurement of antibody levels with the enzyme-linked immunosorbent assay

Diphtheria toxoid-specific IgG, IgA antibody titers in serum, washing fluids of the lung and nasal cavity, saliva and feces were estimated using an ELISA as described previously [5–7]. DT-specific serum IgE antibody titers were determined with a monoclonal antibody-captured ELISA using a commercial anti-mouse IgE antibody (BD Biosciences Pharmingen), and biotin labeled DT and streptavidin-horseradish peroxidase conjugate, prepared with a commercial kit (ECLTM Protein Biotinylation Module, Amersham Biosciences, UK). The enzyme reaction of peroxidase was stopped by adding H₂SO₄. Anti-DT antibody titers were expressed as the highest endpoint dilution of each sample giving a positive reaction and shown as the geometric mean (GM) ± S.D.

2.10. Determination of diphtheria antitoxin titers

The titration of the mouse serum diphtheria antitoxin was carried out using the microcell culture method with VERO cells as described by Miyamura et al. [29] and diphtheria antitoxin titers were expressed as international units (IU) ml⁻¹.

2.11. Statistical analysis

Analysis of antibody titers was performed on logarithmically transformed data and S.D. values were calculated. Comparison of two groups on specific antibody responses were made with unpaired *t*-test, where *p*-values of <0.05 were considered to be statistically significant. Comparison of more than two groups performed by an analysis of variance (ANOVA) test, followed by a post hoc Tukey's or Dunnett's test.

3. Results

3.1. DTH of guinea pigs to PPD or DT

DTH was assessed based on the skin reactions of guinea pigs after intranasal administration of BCG with and without Oligo B. No significant induration was observed in the guinea pigs intranasally vaccinated with BCG (10⁵ CFU), while skin reactions to PPD were potentiated in the guinea pigs intranasally co-administered BCG (10⁵ CFU) and 50 µg of Oligo B in 50 µl saline, with a mean reaction size of 12.9 ± 3.0 mm at 0.2 µg of PPD as shown in Fig. 1. These strong skin reactions were almost the same as that of the subcutaneous administration of BCG at the same dose. Oligo B alone, PPD plus oligo B and methylated oligo B were examined as negative controls, and DTH for methylated oligo B manifested by a mean reaction size of 2.0 ± 2.8 mm, while no DTH was observed for the other substances.

DTH of guinea pigs to DT was not observed at 2 µg of DT. On the other hand, guinea pig anti-DT IgG antibody titers detected by ELISA

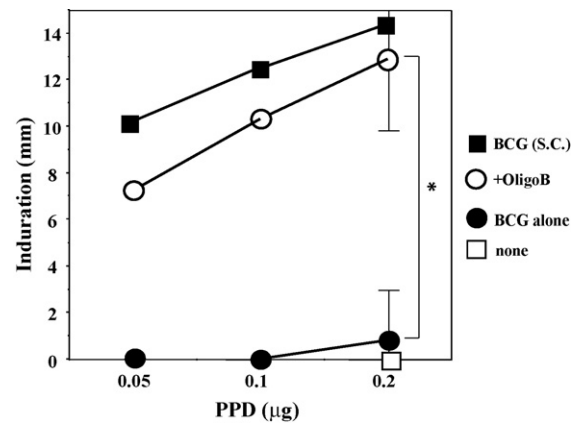


Fig. 1. Effects of rCTB on guinea pig skin reaction to PPD (0.05, 0.1, 0.2 µg), 6 weeks after intranasal (i.n.) BCG vaccination with and without 50 µg CpG in 50 µl saline, as indicated by the induration diameter. Positive control for skin reaction: subcutaneous (s.c.) administration of BCG. The bars indicate standard deviations. The asterisk indicates statistically significant difference at *p* < 0.05 as determined by analysis of variance (ANOVA), followed by a post hoc Tukey's test.

were transformed into logarithms to base 10. Serum IgG antibody titers which were immunized with DT plus Oligo B and DT alone were 5.13 ± 0.16 and 2.43 ± 0.28, respectively.

3.2. Responses of guinea pig or mouse spleen cells to PPD

Stimulation of TNF-α production from the spleen cells by PPD was examined. BCG (10⁵ CFU) vaccination in the presence of Oligo B significantly enhanced the ability of the guinea pig spleen cells to produce TNF-α in response to PPD, as compared to the vaccination without Oligo B (Fig. 2). Similarly, IFN production and proliferation of spleen cells following stimulation with 10 µg/ml PPD were enhanced in guinea pigs vaccinated intranasally with BCG and Oligo B.

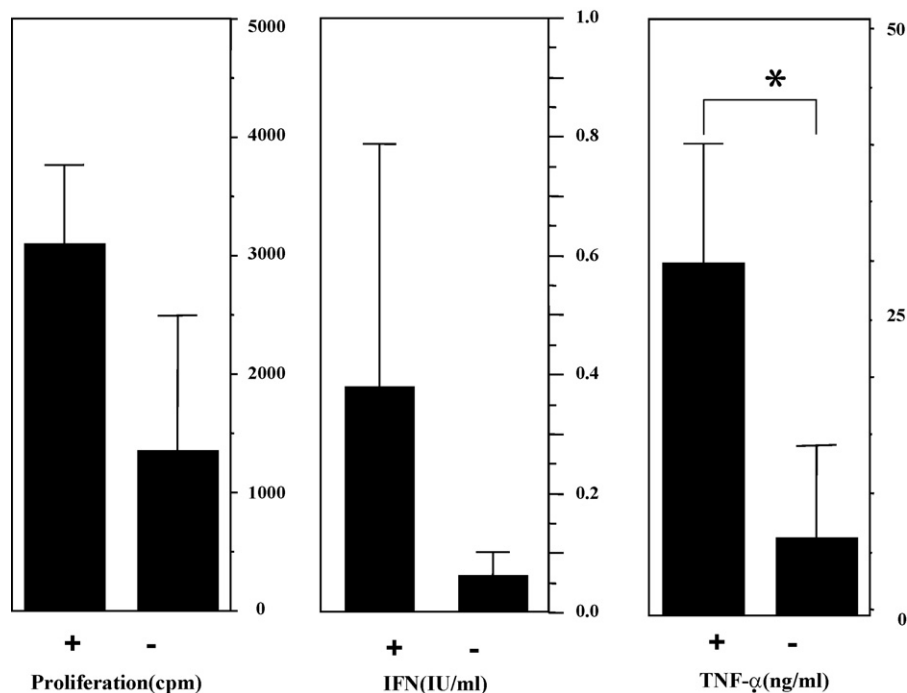


Fig. 2. Effect of Oligo B on Proliferation, IFN, TNF-α production by guinea pig spleen cells stimulated with PPD (10 µg/ml) after intranasal BCG vaccination with or without Oligo B. Asterisk indicates that the mean value in the presence of Oligo B was significantly higher than that in the absence of Oligo B (*p* < 0.05).

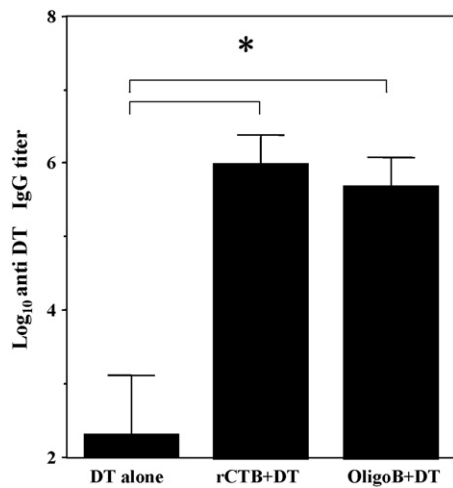


Fig. 3. Serum IgG antibody responses to DT co-administered intranasally with Oligo B (200 μ g/mouse) and rCTB (10 μ g/mouse). IgG antibody titers detected by ELISA were transformed into logarithms to base 10. Asterisk indicates that the mean value in the presence of adjuvants was significantly higher than that in the absence of adjuvants. * $p < 0.05$, as determined by ANOVA, followed by a post hoc Tukey's test.

3.3. Effects of CpG-DNA on antigen-specific serum antibody responses

We first investigated whether Oligo B had mucosal adjuvant activity for soluble protein antigen at the maximum dose possible to prepare. IgG antibody titers detected by ELISA were transformed into logarithms to base 10. Serum IgG antibody titers for BALB/c mice which were immunized with DT alone, DT plus rCTB and DT plus Oligo B were 1.7 ± 1.1 , 6.0 ± 0.4 and 5.7 ± 0.4 , respectively. Intranasal administration of DT plus Oligo B induced high levels of IgG antibody responses, almost the same level as DT plus rCTB (Fig. 3). When the serum IgG subclass responses to DT were assessed, the response of several subclass of serum IgG to DT in the presence of adjuvants was significantly higher than that in the absence of adjuvants, and the major subclass of serum IgG induced in response to DT was IgG1, the same as the case for rCTB.

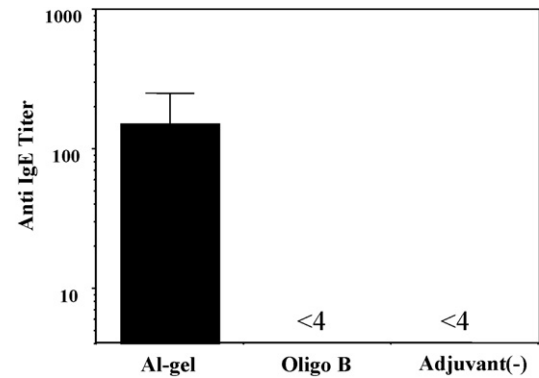


Fig. 5. Serum anti-DT IgE antibody responses to DT coadministered intranasally with Oligo B. The positive control of serum anti-DT IgE antibody responses is the group using the Al adjuvant.

Significantly greater IgG2a and IgG2c antibody responses in the C57BL/6 mice were detected in the presence of Oligo B as compared with those to rCTB (Fig. 4). Intramuscular administration of alum-adjuvant DT provided detected amounts of DT-specific serum IgE, while no DT-specific serum IgE antibodies were detected when administered with Oligo B (Fig. 5). Methylated Oligo B was used for the negative control of the CpG motif. Serum IgG responses to DT administered with Oligo B at a dose of 5 μ g/mouse was significantly higher than that with methylated Oligo B at the same dose. Production of anti-DT antibodies was however observed when methylated Oligo B was administered at a dose of 50 μ g/mouse (Fig. 6). Moreover, preliminary experiment using TLR9 knock out mouse showed that there is no statistically significance between antibody titers of DT with Oligo B and DT alone (data not shown).

Next, we examined the effects of doses of Oligo B on serum antibody responses to intranasally administered DT to determine the minimum concentrations of Oligo B necessary for sufficient induction of serum anti-DT-specific antibody responses (Fig. 7). Intranasal administration of DT in the absence of Oligo B cause a little DT-specific serum IgG antibody response at a concentration of 5 Lf. On the other hand, in the presence of Oligo B above

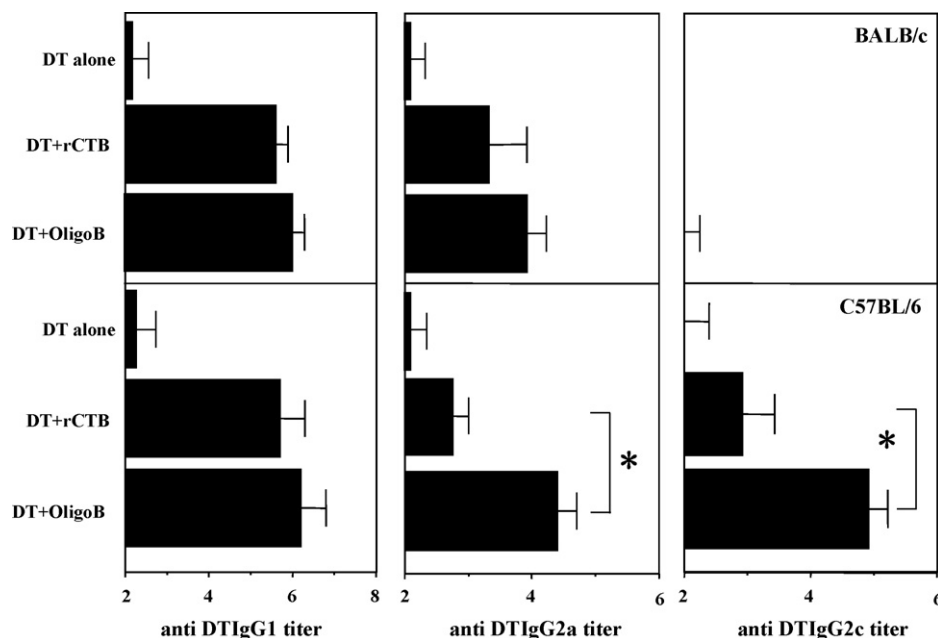


Fig. 4. Serum anti-DT IgG1, IgG2a and IgG2c responses to DT co-administered intranasally with Oligo B. Asterisks indicate that the mean value in the presence of Oligo B was significantly higher than that in the absence of Oligo B. * $p < 0.05$, as determined by ANOVA, followed by a post hoc Tukey's test.

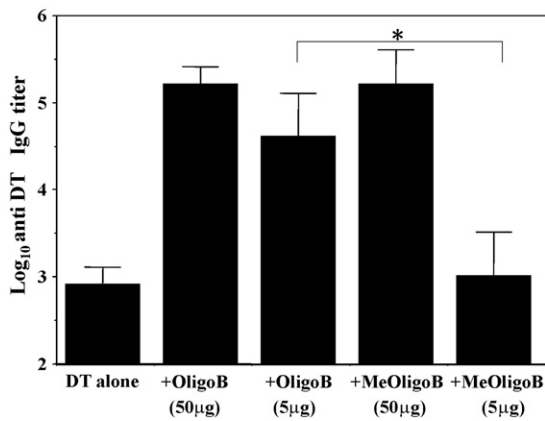


Fig. 6. Serum IgG antibody responses to DT co-administered intranasally with Oligo B (5, 50 μg/mouse) and methylated Oligo B (5, 50 μg/mouse). * $p < 0.05$, as determined by ANOVA, followed by a post hoc Tukey's test.

5 μg/mouse, the nasal administration of DT induced high titers of antigen-specific serum IgG antibodies (Fig. 7A). DT-specific serum IgA antibody was induced in the same manner as that of serum IgG (Fig. 7B).

3.4. Induction of diphtheria antitoxin activity by intranasal immunization with DT in the presence of CpG-DNA

It is generally considered that an antitoxin titer of 0.1 IU ml⁻¹ is the smallest level necessary to protect human subjects from a diphtheria toxin challenge. We therefore measured the antitoxin titers for mice, which were intranasally immunized with 5 Lf DT plus Oligo B (0.1–50 μg/mouse). As shown in Fig. 8, using the same sample as in Fig. 7, mice which indicated high levels of DT-specific serum IgG antibody titers gave sufficiently high antitoxin titers greater than 0.1 IU ml⁻¹. In C57BL/6 and BALB/c, all mice demonstrated sufficiently high antitoxin titers greater than 0.1 IU ml⁻¹ when Oligo B was administered above 5 and 20 μg/mouse (Fig. 8) respectively. These results clearly demonstrate the phylacagogic effect of intranasal administration of DT with Oligo B.

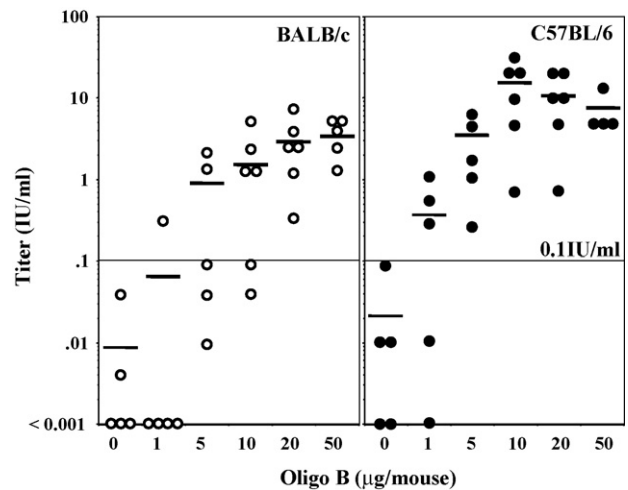


Fig. 8. Serum antitoxin titers of mice intranasally immunized with 5 Lf/mouse DT with Oligo B (0–50 μg/mouse). Each symbol represents a mouse in all the figures. Each bar indicates the mean value.

3.5. Effects on mucosal immune responses to intranasally administered DT with and without CpG-DNA

Intranasal administration of DT without Oligo B induced no or little mucosal IgA responses to DT at the murine mucosal sites examined. DT-specific mucosal IgA antibody titers of 16-fold dilution and over were detected in the nasal cavities, the lung wash and the feces. Lower levels of DT-specific mucosal IgA antibody responses were observed in the saliva, but a significant difference was observed compared with DT without Oligo B (Fig. 9). Neutralizing antibodies in the mucosal secretions were not detected using the microcell culture method with VERO cells.

3.5.1. Cytokine production by mouse spleen cells restimulated with DT after intranasal co-administration of DT plus CpG-DNA

C57BL/6 and BALB/c mice were intranasally immunized with 5 Lf/mouse DT plus 10 μg/ml CpG-DNA, as shown in Fig. 7. Secretion of some cytokines by the mouse spleen cells was assayed after restimulation with DT to examine the effect of CpG-DNA on

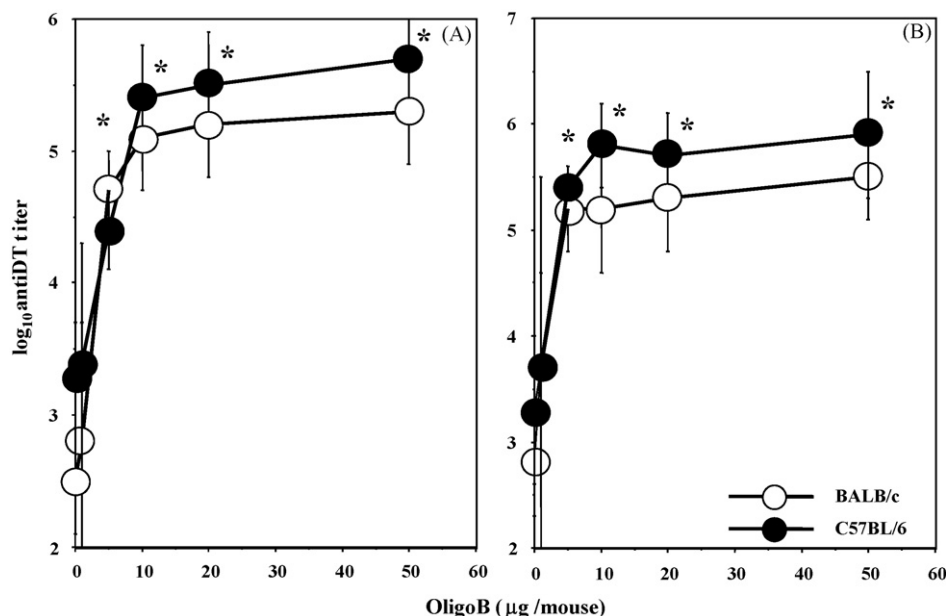


Fig. 7. Dose-dependency of Oligo B on serum anti-DT IgG (A) and IgA (B) antibody production. * $p < 0.05$, as determined by ANOVA, followed by a post hoc Dunnett's test.

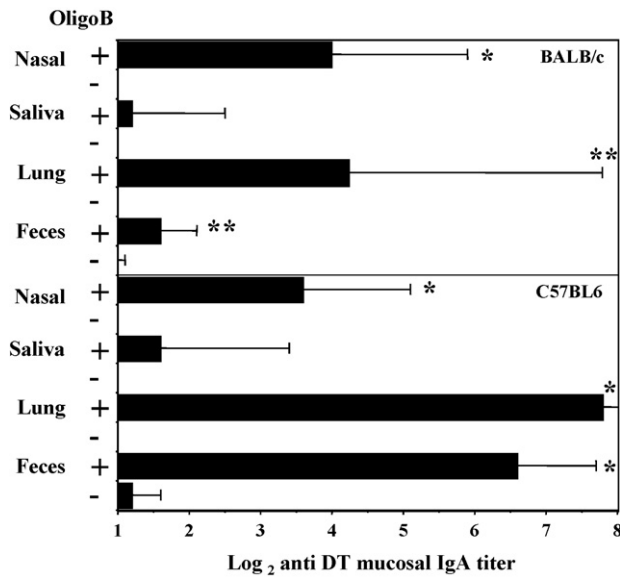


Fig. 9. Mucosal anti-DT IgA antibody responses with or without Oligo B. Mice were immunized intranasally with 5 Lf/mouse DT with or without 10 µg Oligo B. Asterisks indicate that the mean value with Oligo B was significantly higher than without Oligo B (* $p < 0.01$, ** $p < 0.05$).

the immune system. IL-5 and IFN γ were detected, as shown in Fig. 10. Secretions of IL-5 from the C57BL/6 mouse spleen cells and IFN γ from the BALB/c mouse spleen cells were significantly higher following re-stimulation with DT than in the absence of DT stimulation. The mean IL-5 production was higher in the C57BL/6 mice than in the BALB/c mice. On the other hand, the production of IFN γ was significantly higher in the BALB/c mice than in the C57BL/6 mice. No definitive results could be obtained on other cytokines.

4. Discussion

Following developments in research into the TLR system, the signal transduction mechanism of CpG-DNA as been elucidated in

recent years and much research is carried out on the application of CpG-DNA, concentrated mainly on treatments of cancers and allergies. Moreover, CpG-DNA is attracting a great deal of attention as a vaccine adjuvant, based on the concept that CpG-DNA enhances the immunoresponse of Th1 type cells, i.e., cellular immunity [16,17,21].

This report showed that CpG-DNA enhanced both cellular and humoral immunity from the standpoint of adjuvanticity. Considering that the development of multivalent or combined vaccines is one of the aims of future vaccine development, we do not think that any one antigen needs to shoulder the responsibility of inducing both cellular immunity and humoral immunity. In other words, we used two vaccine antigens, that is, DT, which mainly induces humoral immunity, and BCG, which mainly induces cellular immunity, when administered alone. both *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae* are spread person-to-person by respiratory droplets, and in general first infect the mucosal surface. There is a previous report on tetanus toxoid using CpG-DNA as mucosal adjuvant [30]. *Clostridium tetani* penetrates through the wound and grows under anaerobic conditions to produce tetanus toxin at the site of infection.

In this report, we described enhancement of immune responses by the addition of CpG-DNA to either vaccine. Sufficient protection against diphtheria would be provided by both humoral and mucosal immunity. Although the currently used BCG is effective against tuberculosis in infants and young children, it is said that effect of BCG does not last into adulthood, therefore, the protective effect of BCG against adult pulmonary tuberculosis varies [31]. Considering that BCG vaccination is administered in childhood, it might be necessary to undertake additional immunization in adulthood to enhance the immunity against tuberculosis. We think that the results of this experiment may provide clues to the development of countermeasures against adult pulmonary tuberculosis. The evaluation was conducted by the measurement of antibody titers (representing humoral immunity) in the mouse for DT, and by measurement of the reaction to PPD (delayed type hypersensitivity) in guinea pigs induced by BCG.

We first examined the DTH reaction to PPD and responses of spleen cells following intranasal administration of BCG with or

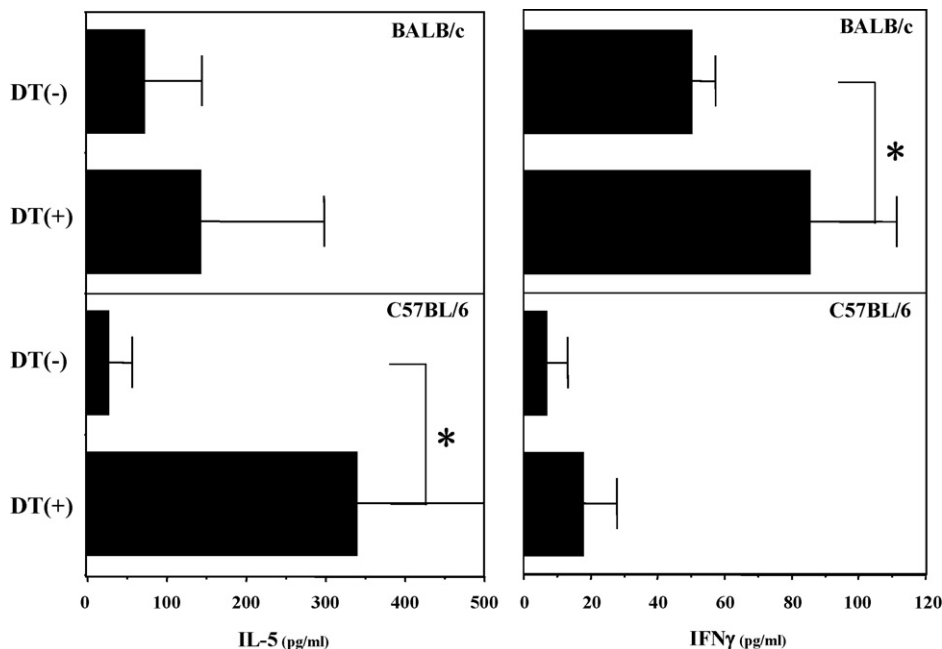


Fig. 10. Cytokine production by mouse spleen cells (1×10^7 cells/ml) incubated with and without DT (10 µg/ml) *in vitro* for 24 h at 37 °C after intranasal co-administration of DT (5 Lf/mouse) and CpG-DNA (10 µg/mouse). (DT+) and (DT-) indicate secondary stimulation *in vitro* with or without DT. * $p < 0.05$, as determined by ANOVA, followed by a post hoc Tukey's test.

without CpG-DNA to confirm the enhancement of cellular immunity by CpG-DNA. Th1 type immune response stimulates cellular immunity, which is not the same as cellular immunity. Therefore, further work is need for investigating Th1 immune responses by detailed Th1-type cytokine measurements.

Next, we examined the mucosal adjuvanticity of CpG-DNA using DT because a method for the evaluation of protection against the onset of diphtheria had already been established [29], and because a soluble protein antigen such as DT has been reported to induce immunoresponse of Th2 type cells when it was administered intranasally [6,25].

The dosage of adjuvant used was 200 µg/mouse at first, the maximum dose that can be prepared. After that, when examining dose-dependency, the ELISA antibody titer showed a high level at 5 µg/mouse. Production of anti-DT antibodies was observed when using methylated Oligo B as a negative control at doses of more than 50 µg/mouse (Fig. 6) and weak DTH was also observed. CpG-DNA was thus suspected to be able to potentiate serum and mucosal antibody production in addition to the CpG motif. DNA is endocytosed into a cellular compartment regardless of the presence or absence of the CpG motif [20]. DNA itself may have some effect on the enhancement of antibody production after cellular uptake. Polyguanosine runs improve cellular uptake and the immunostimulative activity of phosphodiester CpG-DNA [20]. In addition to this effect, 12mer polyguanosine at both the 3' and 5' end of oligo DNA may have adjuvanticity on antibody production. Though mechanisms of adjuvanticity must be made clear in the future, Oligo B certainly enhanced serum and mucosal antibody production, which there was a significant difference compared with the no adjuvant. Research on the molecular mechanism of CpG-DNA, also, has been proceeding in the human plasmacytoid dendritic cells [32–34]. We then measured the antitoxin titers to estimate the protective effect. Titers in all C57BL/6 mice in which CpG-DNA 5 µg/mouse and over was administered, were shown to be more than 0.1 IU/ml. In all BALB/c mice, when the dosage of CpG-DNA was 20 µg/mouse and over, titers in excess of 0.1 IU/ml were observed. These antitoxin titer were sufficiently protective when compared with using rCTB as a mucosal adjuvant [6]. Moreover, we examined mucosal secretions for the presence of neutralizing antibodies. However, we could not obtain definite results, perhaps because of dilution by the washing solutions and also the cytotoxicity of the washing solutions. The current diphtheria toxoid provides protection against the onset of disease. As for mucosal vaccine, the antigen, which should be neutralized on mucosal surface, might be the factors involved in the infection to mucosal membrane rather than diphtheria toxin.

Several studies have focused on the differences in the immune responses to BCG between the BALB/c and C57BL/6 mice [35,36]. The immune responses to the *M. tuberculosis* in the C57BL/6 mice are supposed to be stronger than those in BALB/c mice [35,36]. By contrast, we expected at first that the BALB/c mice would show higher antibody titers against soluble protein antigens than their C57BL/6 counterparts. However, the titers were slightly higher in the C57BL/6 mice than in the BALB/c mice (Figs. 7–9). These data indicate that humoral immune responses are induced variously depending on the pathogen, antigen, adjuvant and strain. There may be some involvement of the difference in the genetic make-up in the responsiveness to antigen and antibody production. IgG2a and IgG2c responses to DT with Oligo B were significantly higher than those observed in the absence of Oligo B, and significant IgG2a and IgG2c antibody responses, involved in Th1-type immunoresponse, were observed in the presence of Oligo B in the C57BL/6 mice as compared with those to rCTB. Production of IgE, an immunoglobulin involved in excessive enhancement of the Th2-type immunoresponse, was not observed at all. Our method may not have been as sensitive as compared to the fluorometric method, but a significant difference was observed as compared with that obtained with alu-

minum gel adjuvant. Under the experimental conditions employed, little IL-4 was detected in the spleen cells from the mice immunized with DT plus Oligo B. In contrast, IL-5 and IFN were clearly detected. Our previous data on rCTB showed production of IL-4 and inhibition of the Th1 response [25]. CpG-DNA has been well-recognized as potentiating the Th1 type of immune responses. It is said that Th1-type immune response stimulates cellular immunity, while the Th2-type immune response stimulates humoral immunity. Further research into the production of cytokines and expression of GATA3 and T-bet is needed to clarify the mechanism of adjuvanticity of CpG-DNA and the relationship between Th1/2 balance and cellular and humoral immunity [37]. In any event, intranasal administration of CpG-DNA potentiated not only cellular immunity, but also humoral and mucosal immunity.

Splenomegaly is a well-recognized phenomenon accompanying the injection of some oligonucleotides. The phosphorothioate backbone CpG-DNA (PS-CpG-DNA) induced particularly massive dose-dependent splenomegaly in a mouse model [38]. However, less splenomegaly was induced by the natural phosphodiester backbone CpG-DNA than by PS-CpG-DNA [20]. Splenomegaly could possibly thus be reduced by reviewing the structure of the CpG-DNA. Also, chronic dosing with PS-CpG-DNA in rodents results in a dose-dependent mononuclear cell infiltration, but such changes do not occur in monkeys and humans [20]. When we consider the large quantities of CpG-DNA administered by injection in the case of cancer and allergy treatment, the response in the case of the small amount required for intranasal administration may not present any problem. Actually, in our preliminary experiments, the number of spleen cells collected from the immunized animals were more than in the non-immunized animals. When compared with rCTB, however, which is a non-toxic adjuvant [39], the number of the spleen-cells were the same in both groups (data not shown). These responses are considered to be in the range of the normal immune response following vaccination.

Our results showed that Oligo B was useful as a mucosal adjuvant from the aspect of phylaxis. For development of new mucosal vaccines, it might be necessary to examine the structure of Oligo B which can be adapted for humans and primates, because our results were obtained in rodent models. Also, it may be necessary to consider whether enhancing the adjuvanticity of Oligo B in a low response group like BALB/c is a good research approach. Moreover, much more research into the aspects of safety will be needed, though, in general, CpG-DNA has been reported to be safe [17,20,21]. However, Oligo B enhanced the immune response regardless of the antigen, which were both a particle live antigen such as BCG and a soluble protein antigen such as DT. Therefore, the use of CpG-DNA as a mucosal adjuvant may well represent a facile approach to the future development of new vaccines for the emerging and reemerging infections. Furthermore, CpG-DNA may be useful in the development of new multivalent vaccines.

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