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Biochemical and Biophysical Research Communications 329 (2005) 230–236
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Resistance to influenza A virus infection by antigen-conjugated CpG oligonucleotides, a novel antigen-specific immunomodulator

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Received 31 December 2004

Abstract

Oligodeoxynucleotides (ODN) containing CpG motifs (CpG) act as modulators that bias the immune response towards a Th1-dominant phenotype. To investigate this effect further, we examined the protective effects of a covalently linked conjugate between CpG-ODN and HA-2kd antigen in mice infected with influenza A virus. The conjugated form of CpG-ODN and HA-2kd was more efficient in regulating influenza A virus than the unconjugated mixture of CpG-ODN and HA-2kd. The antigen-conjugated CpG-ODN induced an immune response with a Th1-dominant cytokine pattern characterized by the secretion of high levels of HA-2kd-specific interferon-γ and IgG2a (Th1), which were only slightly induced by HA-2kd alone. These findings support the use of CpG-ODN-Ag conjugates as novel Ag-specific immunomodulators and suggest that CpG-ODN-HA-2kd might be a promising immune therapy for patients with influenza virus.

Keywords: Oligonucleotides; Antigen-conjugated oligonucleotides; Immunostimulatory sequences; Influenza virus; Hemagglutinin; Th1

While bacterial DNA activates B cells, natural killer (NK) cells, and monocytes, vertebrate DNA does not [1–4]. Injection of synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs (CpG-ODN) in mice results in the activation of antigen-presenting cells that promote interferon (IFN)-γ production by T cells as well as the induction of antigen-specific cytotoxic T lymphocytes (CTL) [5–7]. CpG-ODN has also been shown to enhance natural killer cell reactivity and positively influence B-cell antibody production [2,3]. In light of these effects, it is no surprise that CpG-ODN have been examined for their efficacy in preventing allergy in animal models [8], for experimental cancer treatment [9], and as vaccine adjuvants [6,10,11]. In particular, the fact that CpG-ODN promote Th1 responses has led to the design of phase I clinical trials of its effectiveness in hepatitis B viral patients [12]. Furthermore, studies in mice infected with Leishmania major suggested that CpG-ODN had therapeutic value for the treatment of infectious diseases by facilitating Th1-mediated immunity [13].

The above data suggested that CpG-ODN might also be valuable as a postexposure immune therapy in viral infections. Results from animal studies showed that CpG-ODN exhibited strong Th1 [14–17] and mucosal [18,19] adjuvanticity to a wide range of antigens that included influenza and hepatitis B viral antigens [11,12,20–23], as well as allergens [24,25]; anti-tumor effects of CpG-ODN have also been reported [26,27]. In this study,

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.01.116

we analyzed the immune effects of antigen-conjugated CpG-ODN therapy in mice that were infected with influenza A virus. Our results showed that antigen-conjugated CpG-ODN inhibited influenza virus infection more efficiently than coadministration of the unconjugated mixture of CpG-ODN and Ag. The inhibition of influenza virus infection by the conjugate was Ag-specific and was associated with the presence of unresponsive Ag-specific Th2 cells in regional lymph nodes. Finally, antigen-conjugated CpG-ODN was found to be a stronger Th1 inducer than the unconjugated mixture.

Materials and methods

Oligopeptide. The influenza virus A (A/PR/8/34:H1N1) epitope peptide H-DYEELREQLSK-OH (114–123) [28] was prepared by an automated solid phase peptide synthesizer (Shimadzu PSSM-8, Kyoto, Japan) using standard Fmoc chemistry. MALDI-TOF MS (Voyager DE, PE Biosystems, Foster City, CA) calcd. 1409.51 found *m*/*z* 1410.61. Retention time on the RPHPLC was 21.19 min.

Conjugation of ODN to peptide. The phosphorothioate oligonucleotide (ODN 2006) [29] was prepared with an automated DNA/RNA synthesizer (Cruachem PS250, Glasgow, UK) using standard cyanoethyl phosphoramidite chemistry. The 5′-end hydroxyl groups of the oligonucleotides were reacted with carbonyldiimidazole (CDI) and 1,2-diaminoethane. A solution of 1,6-diisocyanatohexane and *N*,*N*-diisopropylethylamine (DIEA) in dry CH_3CN was injected into the reaction column using a syringe. After 12 h, the CPG support was washed with dry CH_3CN to remove excess diisocyanate. A solution of H-DYEELREQLSK-OH and DIEA in *N*,*N*-dimethylformamide (DMF) was then injected into the reaction column. The products were characterized and confirmed by MALDI-TOF MS. 3′-TTGCTGTTTT-GCTGTTTTGCTGCT-5′-OCONH$(CH_2)_2$NHCONH$(CH_2)_6$NHCNH-ε-K(COOH)SLQERLEEYD(NH_2): MALDI-TOF MS calcd. 9333.70, found *m*/*z* 9355.81. Retention time on the RPHPLC was 34.57 min. 3′-TTCGTGTTTTCGTGTTTTCGTCGT-5′-OCONH$(CH_2)_2$NHCO-NH$(CH_2)_6$NHCO-NH-ε-K(COOH)SLQERLEEYD(NH_2) (control): MALDI-TOF MS calcd. 9333.70, found *m*/*z* 9341.13. Retention time on the RPHPLC was 35.63 min.

Influenza virus. Influenza virus A/PR/8/34 (H1N1) was grown for 3 days in Madin–Darby canine kidney cells cultured in Dulbecco's modified Eagle's medium (Sigma–Aldrich, St. Louis, MO) containing glucose (1000 mg/L), L-glutamine (3 μg/ml), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% (v/v) heat-inactivated fetal bovine serum. Culture supernatants were harvested and stored at −80 °C. The virus titer was determined using a plaque assay, as previously described [30]. To determine the virus titer in the lungs, the mice were sacrificed under light diethyl ether anesthesia [31] and lung homogenates were prepared and serially diluted in saline; viral titers were determined by plaque assays.

Immunization and challenge. BALB/c female mice (6 week old), obtained from Charles River Japan (Kanagawa, Japan), were maintained on a 12-h light–dark cycle. All groups of mice were immunized with HA-2kd and CpG-ODN (5 nmol)) alone, or CpG-ODN-HA-2kd (5 nmol) or CpG-ODN + HA-2kd (5 nmol), respectively, using phosphate-buffered saline (PBS) as a control. Reagents were applied at 15 μl (PBS); each mouse was lightly anesthetized with diethyl ether and was injected intraperitoneally (i.p.). All mice were immunized twice, with a 2-week interval between their primary and booster immunizations. Animals were challenged with a lethal dose of 5.6×10^5 pfu of mouse-adapted A/PR/8/34 influenza virus (100 LD_{50}) in 50 μl saline intranasally 2 week after their second immunization. This infection method induced rapid-widespread viral replication in the lung and death in 5–7 days. The survival rates of mice immunized with the antigen-conjugated ODN and the unconjugated mixture of antigen and ODN were compared.

Antibody assay. The titer of antibody directed against HA was measured in the serum of our animals using an enzyme-linked immunosorbent assay (ELISA). The ELISA was performed sequentially from the solid phase (96-well Nunc Maxisorp P/N, Nalge Nunc International, Rochester, NY) with a ladder of reagents consisting sequentially of the following: HA molecules purified from the A/PR/8/34 influenza virus, a serum sample, goat anti-mouse IgG1, IgG2a, and IgG2b (KPL, Gaithersburg, MD) conjugated with biotin, streptavidin-conjugated with alkaline phosphatase (KPL), and *p*-nitrophenyl-phosphate (Moss, Pasadena, MD). The absorbance of the chromogen was measured at 405 nm in a microplate autoreader (Titertec Multican Labsystem Oy, Helsinki, Finland).

Production of inflammatory cytokines. The concentration of the inflammatory cytokine, interleukin (IL)-6, in lung homogenates was determined by sandwich ELISA using an OptEIA mouse IL-6 Set (BD PharMingen, San Diego, CA). A 96-well ELISA plate (Nunc Maxisorp P/N, Nalge Nunc International) was coated with anti-mouse IL-6 monoclonal antibody, diluted in coating buffer (0.2 M sodium phosphate, pH 6.5) overnight at 4 °C. After washing with PBS containing 0.05% Tween 20 and blocking with PBS containing 10% fetal bovine serum for 2 h at room temperature, a 100 μl aliquot of 10-fold diluted lung homogenate or serially diluted mouse recombinant IL-6 was placed in each well, after which the plates were incubated for 2 h at room temperature. After washing, a total of 100 μl of biotinylated mouse IL-6 monoclonal antibody and avidin-horseradish peroxidase (HRP) conjugate was placed into each well, and the plates were incubated for an additional 1 h at room temperature. After extensive washing, 100 μl of substrate solution was added to, and left in the wells for 30 min at room temperature. Finally, the optical density (OD) of each sample was measured at 450–540 nm in a microplate reader (Titertec Multican Labsystem) following the addition of 50 μl stop solution (1 M H_3PO_4). The concentration of IL-6 was calculated using a standard curve of mouse recombinant IL-6, with substrate solution used as the blank.

Staining for the presence of intracellular IFN-γ and flow cytometry analyses. Splenocytes from naïve and vaccinated mice were incubated with or without HA peptide (HA-2kd; 4 μg/ml) for 24 h at 37 °C in a 5% CO_2 incubator. Brefeldin A (Sigma–Aldrich; final density 10 μg/ml) was added to the cultures, after which the cells were incubated for an additional 6 h. The cells were then washed once in fluorescence-activated cell sorter buffer and stained with 1 μg/10 μl of phycoerythrin-conjugated monoclonal anti-mouse CD^{8+} antibody (Becton–Dickinson Immunocytometry Systems, Mountain View, CA). The cells were also stained for the presence of IFN-γ using a fluorescein isothiocyanate-labeled anti-IFN-γ antibody (IFN-γ-FITC; Becton–Dickinson Immunocytometry Systems). A Becton–Dickinson FACScan with the CELLQuest Software (Becton–Dickinson Immunocytometry Systems) was used for all of the analyses.

Cytokine ELISPOT assay. The ELISPOT assay was performed using a Mouse Cytokine ELISPOT Set (IFN-γ, IL-4) (Becton–Dickinson Immunocytometry Systems) [32]. Splenocytes (20×10^4 cells/well in 100 μl) in complete medium, with or without HA-2kd, were incubated in the assay wells at 37 °C for 24 h in a 5% CO_2 incubator. The wells were then washed six times with PBS containing 0.025% Tween 20, after which they were treated with 50 μl (5 μg/ml) of the anti-mouse cytokine (biotinylated anti-mouse-IFN-γ and IL-4) ELISPOT Set. After a final wash with PBS, the IFN-γ and IL-4 spot forming cells were detected by the addition of a BCIP-nitroblue tetrazolium solution and counted using a stereomicroscope.

Histopathologic examination. Mice were sacrificed under light inhaled diethyl ether anesthesia on day 7 following influenza virus infection. Their lungs were removed and inflated with 10% formalin in PBS. After fixation, the lungs were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Statistics. Data are shown as means ± standard error (SEM). A Student's *t* test was used to compared virus titers and cytokine production between groups.

Results

HA antigen-conjugated CpG-ODN induced the production of Th1-associated antigen-specific IgG2a

In this study, we injected mice intraperitoneally (i.p.) with the antigen (influenza A virus [A/PR/8/34] 114–123, DYEELREQLS, HA-2kd [28])-conjugated CpG-ODN (CpG-ODN-HA-2kd) or the unconjugated mixture CpG-ODN (CpG-ODN + HA-2kd) to examine the IgG sub-classes (IgG1, IgG2a, and IgG2b) produced in serum, and evaluated the immune effect for the mice. BALB/c mice were injected intraperitoneally with 5 nmol of oligonucleotides and HA-2kd twice, with a 2-week interval. Serum samples were collected and then assayed for the anti-IgG sub-class by ELISA (Fig. 1). In comparison to saline-treated control, immunization of mice with the CpG-2-ODN + HA-2kd induced slightly more anti-IgG2a antibodies. Surprisingly, the failure to produce higher levels of IgG2a with the CpG-ODN + HA-2kd was alleviated by directly linking the two components; the antigen-conjugated CpG-ODN (CpG-ODN-HA-2kd) induced higher levels of IgG2a [33]. On the other hand, mice injected with HA-2kd alone induced low levels of IgG2a. Mice that were immunized with a mixture of non-CpG-ODN (GpC-ODN) and HA-2kd (GpC-ODN + HA-2kd) or antigen-conjugated non-CpG-ODN (GpC-ODN-HA-2kd) also produced only low levels of IgG2a. There were no significant changes in the IgG1 and IgG2b levels in all of the above mice (Fig. 1) [23].

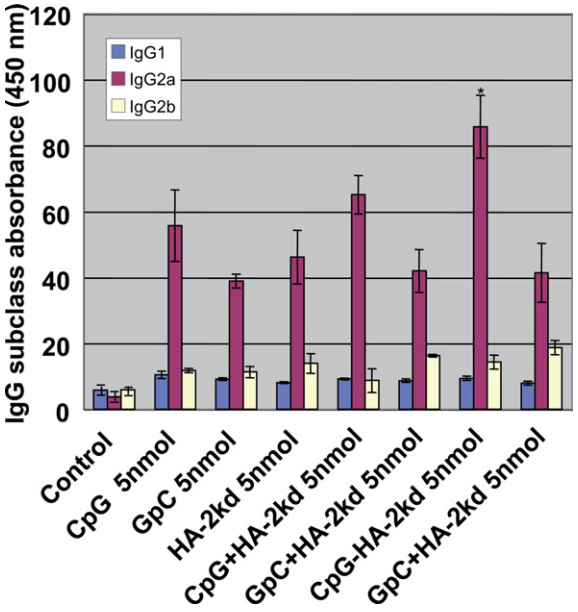

Fig. 1. Th1-associated antigen-specific IgG2a responses were induced by immunization of BALB/c mice with antigen-conjugated CpG-ODN or the antigen-conjugated mixture CpG-ODN. Mice were immunized with an i.p. injection of HA-2kd, CpG-ODN-HA-2kd, CpG-ODN + HA-2kd, GpC-ODN-HA-2kd, or GpC-ODN + HA-2kd on days 0 and 14. Two weeks later, serum antibody titers were measured. Each group consisted of eight mice, and experiments were carried out twice. $^{*}p < 0.05$ compared to CpG-ODN + HA-2kd.

Staining for the presence of intracellular IFN-γ and flow cytometry analyses

We therefore assessed the number of influenza A virus HA-specific CD^{8+} T cells expressing IFN-γ in the spleens of mice immunized with the antigen-conjugated CpG-ODN. Intracellular cytokine staining and ELISPOT [32] are sensitive functional assays used to measure IFN-γ at the single-cell level. We observed high numbers of CD^{8+} cells that stained for the presence of intracellular IFN-γ in the spleens of mice immunized with CpG-ODN-HA-2kd or CpG-ODN + HA-2kd compared to mice immunized with HA-2dk (Fig. 2). Furthermore, the results of the ELISPOT assay revealed higher levels of IFN-γ in the spleen cells of mice immunized with CpG-ODN-HA-2kd compared to those of mice immunized with HA-2kd (Fig. 3). On the other hand, the levels of IL-4 were below the level of detection (data not shown).

Protection from lethal challenge with influenza virus in mice immunized with CpG-ODN-HA-2kd

The goal of immunization with influenza virus antigen is to inhibit influenza virus replication. To determine the immunostimulatory effects induced by CpG-ODN-HA-2kd in more detail, the protection from the influenza virus infection was compared to those induced by treatments with HA-2kd and CpG-ODN + HA-2kd. The virus titer and the inflammatory cytokines within the lungs 3 days post-challenge were determined to assess the level of protection of the mice from an acute lung influenza infection. Mice were immunized twice, 2 weeks apart, with antigen-conjugated CpG-ODN-HA-2kd and an unconjugated mixture, CpG-ODN + HA-2kd, intraperitoneally. Two weeks after their second immunization, the mice were challenged with a lethal dose (5.6×10^5 pfu) of influenza virus. Mice inoculated with CpG-ODN-HA-2kd were completely protected following lethal challenge with influenza virus and displayed only minimal weight loss and no changes in activity and grooming (Fig. 4). In contrast, mice immunized with CpG-ODN + HA-2kd or HA-2kd showed 80% and 60% survival, respectively. Furthermore, mice immunized with CpG-ODN-HA-2kd had significantly reduced virus titers and IL-6 production in their lungs compared to those immunized with CpG-ODN +

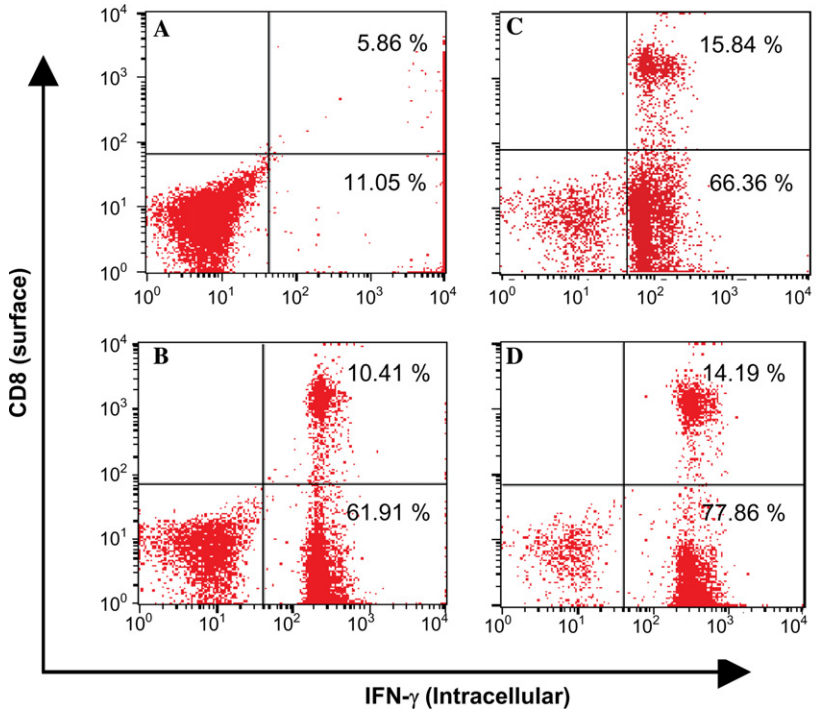

Fig. 2. Intracellular cytokine staining and flow cytometry analysis. Mice were immunized with CpG-ODN-HA-2kd or CpG-ODN + HA-2kd on days 0 and 14. Two weeks later, their splenocytes were incubated with or without HA peptide (HA-2kd) for 24 h, after which they were stained for both CD8 and intracellular IFN-γ. (A) Saline; (B) HA-2kd (5 nmol); (C) CpG-ODN + HA-2kd (5 nmol); and (D) CpG-ODN-HA-2kd (5 nmol).

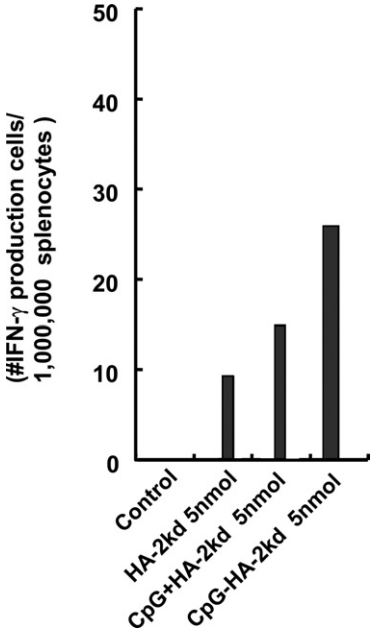

Fig. 3. ELISPOT assay. Mice were immunized i.p. with CpG-ODN-HA-2kd or CpG-ODN + HA-2kd on days 0 and 14. Two weeks later, their splenocytes were incubated with or without HA peptide (HA-2kd) for 24 h, after which the ELISPOT assay was performed. Experiments were repeated three times with similar results.

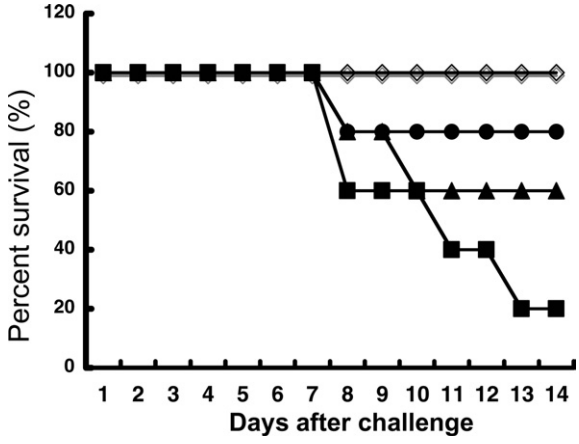

Fig. 4. Protection from a lethal challenge of influenza virus. Mice were immunized i.p. with HA-2kd (▲), CpG-ODN-HA-2kd (◊), or CpG-ODN + HA-2kd (●). Control mice were inoculated with saline (■). Two weeks after their second immunization, the mice were challenged with a lethal dose (5.6×10^5 pfu) of influenza virus. Survival rates were recorded through 14 days post-challenge.

HA-2kd (Figs. 5A and B) [33]. Immunization of mice with CpG-ODN + HA-2kd did not significantly alter their IL-6 levels (Fig. 5B), while mice injected with GpC-ODN + HA-2kd or GpC-ODN-HA-2kd displayed the same levels of virus titers and IL-6 production as the infection controls.

The histologic changes in the lungs of mice challenged with influenza virus after they received an i.p. inoculation with CpG-ODN-HA-2kd are shown in Fig. 6. Neither inflammatory cells nor damaged tissues were observed in the lungs of naïve controls (Figs. 6A-1 and 2). On

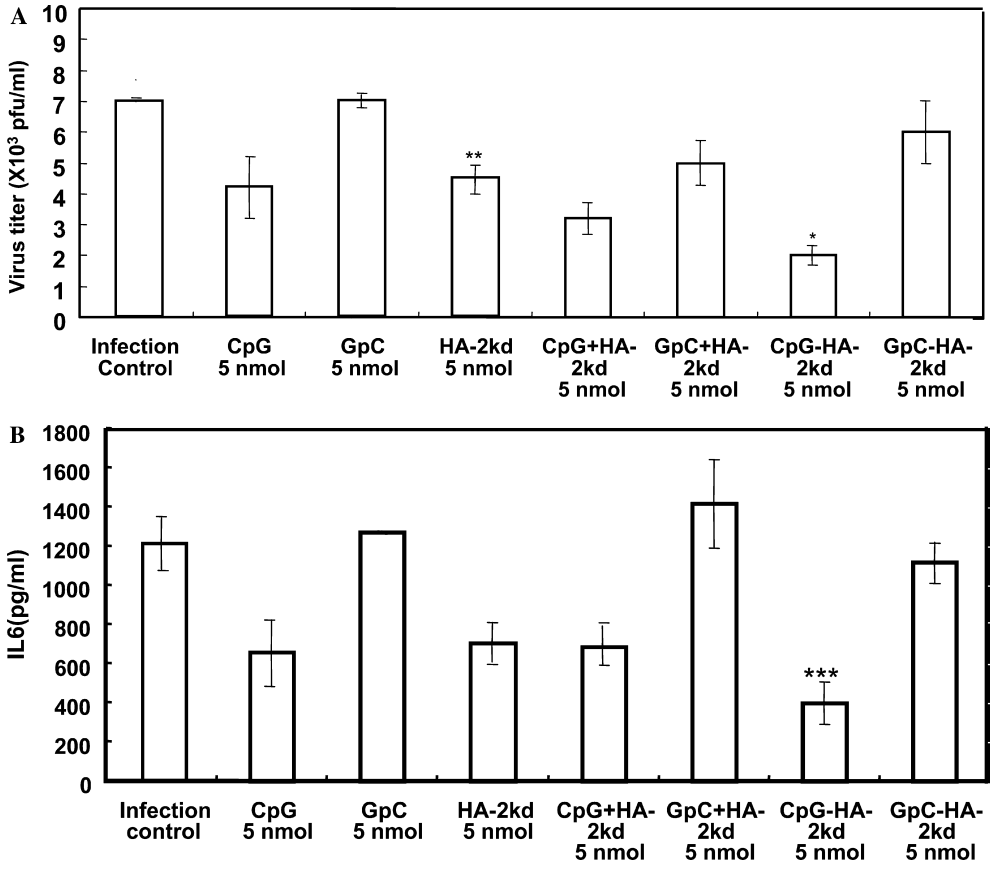

Fig. 5. Virus titers (A) and the concentrations of inflammatory cytokines (B) in the lungs of influenza virus challenged mice that were immunized i.p. with HA-2kd, CpG-ODN-HA-2kd, CpG-ODN + HA-2kd, GpC-ODN-HA-2kd, or GpC-ODN + HA-2kd on days 0 and 14. Two weeks later, the mice were challenged with a lethal dose of influenza virus. On day 31 (3 days after challenge), the virus titers were examined in lung homogenates. Each group consisted of eight mice, and experiments were carried out twice. *$p < 0.01$ compared to CpG-ODN + HA-2kd. **$p < 0.001$ compared to HA-2kd. ***$p < 0.01$ compared to CpG-ODN + CpG-ODN + HA-2kd.

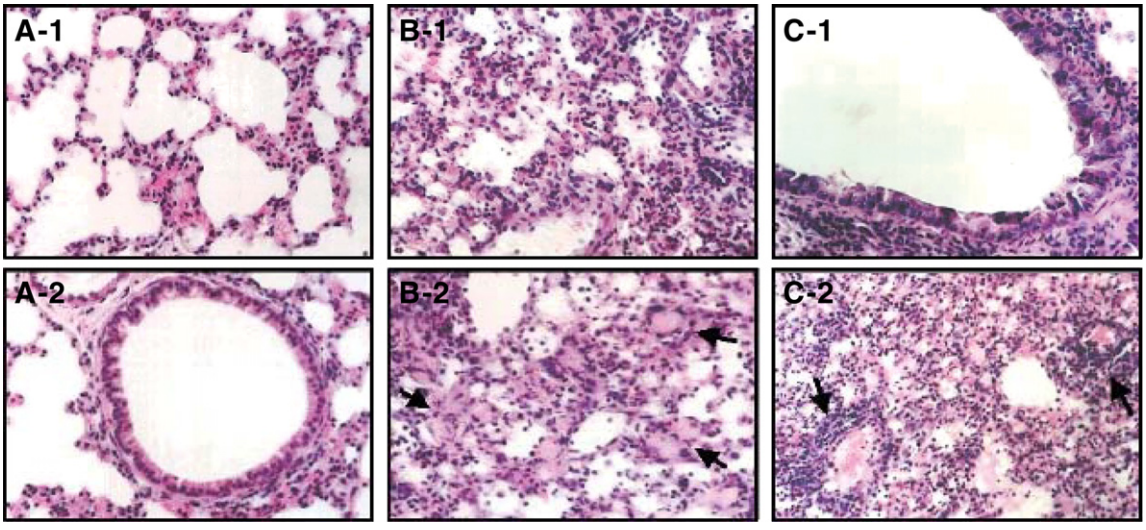

Fig. 6. Effects of pretreatment with CpG-ODN-HA-2kd on lung morphology in mice infected with influenza virus. Seven days after influenza challenge, lungs were removed, sectioned, and stained with hematoxylin and eosin. (A) Uninfected control lung. (B) The lung of an untreated mouse infected with influenza virus displayed mononuclear cell infiltration and the presence of polymorphonuclear cells and hydatid cambium within the perialveolar space (arrow). (C) The lung of a mouse that was pretreated with CpG-ODN-HA-2kd prior to being infected with influenza virus exhibited marked inflammatory cell infiltration within the perialveolar spaces and perivascular lymphocytic infiltration, but exhibited less lung damage than untreated, virus-infected animals.

the other hand, untreated mice infected with influenza virus displayed neutrophil infiltration and marked congestion within the peribronchiolar and perialveolar spaces (Fig. 6B-1). Moreover, mononuclear and polymorphonuclear cell infiltration, as well as hydatid cambium, was observed within the perialveolar space (Fig. 6B-2). In contrast, mice pre-inoculated with CpG-ODN-HA-2kd exhibited marked inflammatory cell infiltration within the perialveolar spaces and perivascular lymphocytic infiltration (Fig. 6C-1), as well as regeneration/hyperformation of their bronchiolar epithelium (Fig. 6C-2). These results suggest that the inflammatory cell and lymphocyte infiltrations were strongly induced by preinoculation with CpG-ODN-HA-2kd, and that these activated immunocompetent cells suppressed the spread of the influenza virus infection in lung tissues.

Discussion

In this study, we evaluated the protective effects of a covalently linked conjugate between CpG-ODN and HA-2kd in mice infected with influenza A virus. The ODN and HA-2kd were conjugated by mixing the carbonyldiimidazole and 1,2-diaminoethane-activated ODN with H-DYEELREQLSK-OH (114–123) [28]. Mice were injected i.p. with HA-2dk-conjugated CpG-ODN (CpG-ODN-HA-2kd) or the unconjugated mixture CpG-ODN (CpG-ODN + HA-2kd) and their serum production of IgG1, IgG2a, and IgG2b was assessed. We found that CpG-ODN-HA-2kd induced higher levels of IgG2a than CpG-ODN + HA-2kd. On the other hand, mice immunized with HA-2kd produced the lowest levels of IgG2a. The IgG1 and IgG2b responses were similar in all groups [23]. These results led us to conclude that antigen-specific IgG2a antibodies were induced by antigen-conjugated CpG-ODN, and further suggested that CpG-ODN induced a Th1-dominant response to the co-administered protein and antigen conjugate [15,16]. CpG-ODN was reported to act as a powerful adjuvant only when it was conjugated with an antigen [5]. The data of Shirota et al. [24] suggested that antigens are picked up, processed, and presented to Th1 and Th2 cells by acyl carrier proteins (ACPs), whereas CpG-ODN promotes the activity of IL-12-secreting APCs, which bias T cell development toward a Th1 phenotype. APCs engulfing antigen-conjugated CpG-ODN present antigenic peptides at the same time that they secrete IL-12. Since antigen-specific Th1 and Th2 cells are presented antigen by IL-12-secreting APCs, IL-12 could act on these cells to promote the preferential activation of the Th2 cells. Antigen-conjugated CpG-ODN induced: (1) greater T-cell responses than did HA-2kd; and (2) a greater number of IFN-γ-producing CD^{8+} T cells after stimulation with MHC class I-restricted HA-2kd-specific peptide and CpG oligonucleotides. There was a good correlation between memory cytotoxic T lymphocyte activity and the number of CpG-ODN-HA-2kd-specific, INF-γ-producing CD^{8+} T cells identified by intracellular cytokine staining. This model should be applicable to other antigens for which the MHC class I epitopes are unclear and should help to evaluate the relative long-term potencies of various vaccine strategies.

To investigate the immunostimulatory effects of CpG-ODN-HA-2kd, we assessed its ability to protect mice from influenza virus infection. Our data showed that virus growth in the lung was markedly inhibited in CpG-ODN-HA-2kd immunized mice, while HA-2kd and CpG-ODN + HA-2kd-treated mice showed no significant reduction in virus. Furthermore, mice immunized with CpG-ODN-HA-2kd had significantly lower levels of IL-6 in their lungs compared to mice immunized with CpG-ODN + HA-2kd. These results suggest that the CpG-ODN-HA-2kd stimulated IL-6 production which helped to reduce lung virus titers [34]. All of the mice pre-treated with CpG-ODN-HA-2kd survived their otherwise-lethal influenza virus infection. Histological examination suggested that CpG-ODN-HA-2kd immunization triggered an inflammatory cell and lymphocytic infiltration that helped to suppress the spread of the influenza virus infection in lungs.

In conclusion, we herein described a novel approach for the control of influenza virus in a mouse model. Our strategy involved the use of antigen-conjugated CpG-ODN, which, when injected into mice effectively enhanced their Th1-type response. The efficient induction of antigen-specific Th1 cells by the systemic administration of CpG might prove to be beneficial not only for the treatment of infection but also cancer.

Acknowledgments

We thank Professor G. Hartmann for his helpful discussions. This work was supported in part by a Grant-in-Aid for High Technology Research, from the Ministry of Education, Science, Sports and Culture, Japan, and a grant from the Japan Society for the Promotion of Science in the "Research for the Future" program (JSPS-RFTF97L00593).

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