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Short communication

CpG oligodeoxynucleotides protect against the 2009 H1N1 pandemic influenza virus infection in a murine model

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ABSTRACT

The 2009 H1N1 influenza virus pandemic poses a global public health threat, and there is a critical need for antiviral drugs for pandemic control. CpG oligodeoxynucleotides have strong immunostimulatory properties and are expected to be used as prophylactic agents to protect against microbial infections. The present study evaluated the efficacy of synthetic CpG oligodeoxynucleotide (ODN) 1826 against pandemic H1N1 virus infection in a murine model. A single injection of 15 μg ODN 1826 intraperitoneally prior to virus challenge inhibits virus replication in lungs, reduces lung lesions and prevents mortality in mice, indicating CpG ODNs as a possible strategy for future influenza pandemics control.

The 2009 H1N1 influenza pandemic poses a global public health threat. According to the World Health Organization (WHO), as of 13 June 2010, at least 18,393 death cases have been reported worldwide (WHO, 2010). Antiviral drugs and vaccines are clearly of great benefit for pandemic control. Licensed anti-influenza drugs such as oseltamivir and zanamivir have been recommended for the management of novel H1N1 infections (WHO, 2009). However, considering the emergence of drug-resistant strains and limited production capability, there is a great need to develop strategies for the prevention and control of the current and next pandemic influenza.

CpG oligodeoxynucleotides (ODNs) are single-stranded sequences that include an unmethylated cytosine–guanosine sequence and certain flanking nucleotides (Krieg, 2006). CpG ODNs can activate toll-like receptor 9 (TLR-9), which subsequently initiates a rapid innate immune response characterized by the secretion of a variety of proinflammatory and antiviral cytokines. Based on the structure and immune properties, CpG ODNs have been identified into 3 classes: A, B and C. A- and B-class CpG ODNs are mainly characterized by induction of high levels of IFN-α secretion or strong B cell activation, respectively, and C-class possess intermediate immune properties between the A and B classes (Krieg, 2006; Vollmer et al., 2004). Previously, CpG ODNs were widely used as effective vaccine adjuvants (Bhat et al., 2010; Dumais et al., 2002; Klinman, 2006; Krieg, 2006; Weiner et al., 1997). Recently, CpG ODNs have been demonstrated to be excellent immune stimulators with the potential to protect against viral infections in a non-specific manner (Ashkar et al., 2003; Dong et al., 2003; Kamstrup et al., 2006; Norton et al., 2010; Rees et al., 2005; Schlaepfer et al., 2004; Wong et al., 2005, 2009). A study in senescence-accelerated model mice revealed that CpG ODNs could induce specific cytotoxic T-lymphocyte responses and natural killer cell activation, protecting against seasonal influenza virus (Dong et al., 2003). CpG ODNs have also been shown to induce local mucosal innate immunity and to inhibit HSV-2 replication (Ashkar et al., 2003). Moreover, the delivery of B class CpG ODNs intranasally could induce the secretion of RANTES and MIP-1β and protect mice against a subsequent intranasal challenge with vaccinia virus (Rees et al., 2005). Other in vivo studies also showed that CpG ODNs offer potential for the prevention of human immunodeficiency virus (HIV), foot and mouth disease virus (FMDV) and seasonal influenza virus challenge (Kamstrup et al., 2006; Norton et al., 2010; Schlaepfer et al., 2004; Wong et al., 2005, 2009). These results suggested that CpG ODNs could provide potent innate protection against both acute and chronic viral infections. Synthetic CpG ODNs exhibited excellent aqueous solubility, stability, standard synthesis and purification procedure and well-characterized absorption, distribution, metabolism and elimination properties, which were quite attractive for drug development and make CpG ODNs excellent drug candidates. ODN 1826, a well-defined B class CpG DNA (Krieg, 2006), has strong immunostimulatory properties in mice, is an adjuvant to vaccines

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against infectious disease or cancer and prevents infection from viruses and bacteria such as FMDV and HSV-2 (Ashkar et al., 2003; Kamstrup et al., 2006).

This study evaluated the efficacy of CpG ODN 1826 to prevent viral replication in the lungs and lethality in mice challenged with the 2009 H1N1 pandemic influenza virus. A murine model of the 2009 H1N1 pandemic influenza virus infection was developed firstly. The 2009 H1N1 pandemic influenza virus A/California/07/2009 (CA07) (GenBank accession nos. FJ966974–FJ966978) was passaged in MDCK cells three times for a titer of 10^8 $TCID_{50}$ (50% tissue culture infective dose)/ml. Mice infected intranasally with 10^7 $TCID_{50}$ (3 LD_{50}) of CA07 virus developed severe symptoms including lethargy, anorexia and severe weight loss and finally died 6–12 days after infection. Virus isolation confirmed that CA07 can replicate in the lungs of infected mice, and pathological examination demonstrated severe lung lesions.

Some previous studies showed that 2009 pandemic influenza viruses such as A/California/04/2009, A/Mexico/4482/2009 and A/Texas/15/2009 virus could replicate efficiently in mouse lungs without prior host adaptation (Itoh et al., 2009; Maines et al., 2009). The mice infected with high dose pandemic H1N1 virus experienced a spectrum of disease parameters including death, weight loss, high lung virus titers, lung lesions and increased lung weight. Our mouse model infected intranasally with 10^7 $TCID_{50}$ of CA07 virus also showed similar pathogenetic characteristics.

Based on this mouse model, the possible protective roles of CpG ODN 1826 were further assayed. Nuclease-resistant phosphorothioate-modified ODN 1826 (5′-TCCATGACGTTCCTGACGTT-3′), was synthesized by Invitrogen (Shanghai) and dissolved in endotoxin-free phosphate-buffered saline (PBS). Four-week-old female Balb/c mice were administered a single injection of 15 μg ODN 1826 intraperitoneally two days before intranasal challenge with 10^7 $TCID_{50}$ CA07 virus under pentobarbital sodium anesthesia (50 mg/kg). Mortality was monitored for 15 days, and survival analyses were performed using a log-rank test. In another set of experiments, four Balb/c mice were administered ODN 1826 or PBS and were infected by virus in the same manner. All infected mice from each group were euthanized on day 5 after infection, and lungs were collected. Part of each lung was fixed in formalin and processed for pathological examination. The remaining part of each lung was weighed, and the lung index was calculated. The lungs were homogenized in 1 ml maintenance media. The virus titrations were determined by end-point titration in MDCK cells. Student's *t*-test was used to determine whether a significant difference existed between the groups tested. All animal experiments with the 2009 H1N1 pandemic influenza virus were performed in accordance with guidelines of the Animal Experiment Committee of the State Key Laboratory of Pathogen and Biosecurity in an animal biosafety level 3 laboratory.

The results demonstrated that 6 of 15 infected mice administered CpG ODN 1826 survived, whereas all the infected mice administered PBS died within 14 days (Fig. 1A). The survival rate (40%) of ODN 1826-treated mice was significantly higher than that of PBS-treated mice ($P < 0.01$, by χ^2 test). Log-rank analysis showed that there was a trend towards improvement from a mean survival of 8.5 days in mice treated with PBS to 11.5 days in mice treated with ODN 1826 (Table 1). In addition, challenge with the 2009 H1N1 virus in mice caused 44.2% mean weight loss by day 12, while only 22.6% mean weight loss was observed in mice treated with ODN 1826 12 days after challenge (Fig. 1B).

The efficacy of CpG ODN 1826 was further tested by assessing its effects on viral replication and lung injury. The viral loads in the lungs and lung index were investigated on day 5 after infection. As shown in Table 1, ODN 1826 pretreatment significantly decreased viral load in the lungs compared with mice given PBS, suggesting that CpG treatment inhibited viral replication in the lungs. The lung

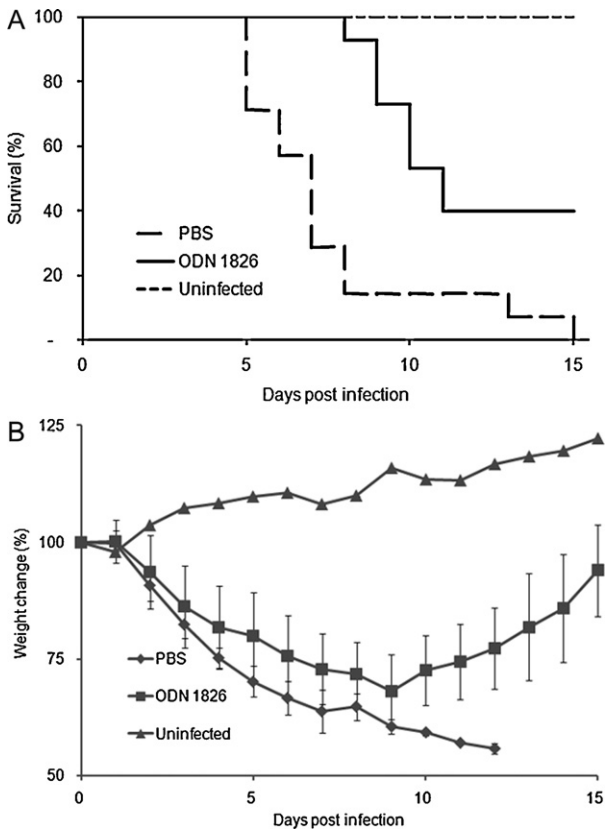

Fig. 1. Protection in mice conferred by ODN 1826 administration. Balb/c mice were treated with a single intraperitoneal injection of ODN 1826 or PBS two days before intranasal challenge with 10^7 $TCID_{50}$ CA07 virus. (A) Survival curves of group of mice treated with ODN 1826 and the control groups after challenge. $P < 0.01$ for ODN 1826 group *vs.* PBS group, by log-rank test. (B) Mean percentage weight loss in groups of mice treated with ODN 1826 or PBS and the uninfected group after challenge.

index of infected mice administered PBS or ODN 1826 increased after challenge in comparison with uninfected mice, whereas a significant difference ($P < 0.05$, by Student's *t*-test) was observed between the ODN 1826 and the PBS group, suggesting the protective benefits of ODN 1826. Pathological examination also revealed that severe lung lesions occurred in infected mice administered PBS (supplemental Fig. 1A and B). Alveolar spaces were occupied by inflammatory infiltrates. Hemorrhage and severe thickening of alveolar walls were also common in lungs of the infected mice. Infected mice pretreated with ODN 1826 had only mild inflammatory infiltrates in the interalveolar septa (supplemental Fig. 1C and D).

Table 1
Protection of CpG ODN 1826 against the 2009 H1N1 pandemic influenza virus.

Treatment	No. of survivors/total no.	Mean survival days	Lung virus titer (log_{10} $TCID_{50}$/g)	Lung index[a] (5(%)d.p.i)
ODN 1826	6/15	11.5 ± 1.1	2.4 ± 2.6[b]	0.93 ± 0.18[c,d]
PBS	0/14	8.5 ± 1.4	6.3 ± 0.1	1.80 ± 0.28
Uninfected	8/8	/	/	0.66 ± 0.10

Balb/c mice were administered a single injection of 15 μg ODN 1826 or PBS intraperitoneally two days before intranasal challenge with 10^7 $TCID_{50}$ CA07 virus. Mortality was monitored for 15 days. Lung samples from four mice of each group were examined on day 5 after infection. Data represent the mean ± SD.

[a] Lung index = lung weight (g)/body weight (g) × 100%.
[b] $P < 0.05$ for ODN 1826 group *vs.* PBS group, by Student's *t* test.
[c] $P < 0.05$ for ODN 1826 group *vs.* uninfected group, by Student's *t* test.
[d] $P < 0.01$ for ODN 1826 group *vs.* PBS group, by Student's *t* test.

This is the first report that CpG ODNs afford protection against the 2009 H1N1 pandemic influenza virus in a murine model. Our data showed that a single dose of 15 μg ODN 1826 two days prior to virus challenge has the ability to inhibit virus replication in lungs, reduce lung lesions and prevent mortality in mice. This is particularly important in view of the great risk of pandemics and the critical need for anti-influenza drugs.

Previously, different groups have shown that pre-treated with a single dose of 1, 5 or 25 μg of CpG ODNs provided partial protection to mice against lethal seasonal influenza virus (Dong et al., 2003; Norton et al., 2010; Wong et al., 2005). While a recent study demonstrated that a single dose of 5 μg of CpG conferred full protection in mice against a mouse-adapted seasonal influenza strain (Wong et al., 2009). Based on these reports, a single dose of 15 μg CpG ODN 1826 was chosen to prevent lethal respiratory pandemic H1N1 influenza virus infections in mice. In addition, CpG ODN has excellent pharmaceutical properties and can be administered via different routes including intranasal, subcutaneous, oral or intraperitoneal. In this study, to achieve systemic delivery of CpG ODNs and induce generic immune response, ODN 1826 was intraperitonealy administrated as previously described (Dong et al., 2003; Rees et al., 2005) and a protective effect was observed.

Further, whether these results in animals can be translated into significant benefits in humans is still unknown, and well-controlled clinical trials are needed to evaluate the prophylactic potential of CpG ODNs against influenza infection. Although previous clinical trials of CpG ODNs have demonstrated substantial clinical benefit for infectious diseases and cancer, there are still many questions that should be answered. Regardless, these results in mice demonstrated that CpG ODN represent a possible strategy in future influenza pandemics control.

Conflict of interests

All authors report no potential conflicts.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2010.11.013.

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