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Original article

Intranasal administration of adjuvant-combined recombinant influenza virus HA vaccine protects mice from the lethal H5N1 virus infection

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Abstract

Attenuated recombinant H5N1 influenza virus was constructed to develop a safe H5N1 influenza vaccine. The immunogenicity and protective effect of the vaccine prepared from haemagglutinin-modified recombinant H5N1 influenza virus was evaluated in mice intranasally co-administered with cholera toxin B subunit containing a trace amount of holotoxin (CTB*), synthetic double-stranded RNA, poly (I:C) or chitin microparticles (CMP) as adjuvants. Intranasal administration of recombinant H5 HA split vaccine with CTB* or poly(I:C) and/or CMP elicited an immunological response with both anti-H5 HA IgA in the nasal wash and anti-H5 HA IgG antibody in the serum, and showed a protective against lethal H5N1 A/Hong Kong/483/97 (HK483) infection. We also demonstrated that intranasal co-administration of antigen with both poly (I:C) and CMP enhanced the expression of Toll-like receptor (TLR) 3, TLR7 in the spleen. These results indicate that poly (I:C) and CMP are highly effective as mucosal adjuvants for use with the nasal H5N1 vaccine.
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Keywords: H5N1; Double-stranded RNA (dsRNA); Poly (I:C); Chitin microparticle (CMP); Adjuvant; IgA; Toll-like receptor (TLR)

1. Introduction

In 1997, the highly pathogenic avian influenza A virus strain, H5N1, transmitted directly from chickens to humans killed 6 of 18 infected people in Hong Kong [1,2]. In 2003–2005, the H5N1 virus caused outbreaks in poultry in East Asian countries, and again transmission of infection from birds to humans resulted in fatal disease [3]. The development of an anti-H5N1 vaccine is the best strategy to prevent a potential human pandemic of this virus. However, it was not possible to prepare a vaccine against the Hong Kong H5N1 virus using the conventional embryonated egg system because of its lethality in chicken embryos [4]. Avirulent recombinant H5N1/PR8 virus for use as a vaccine was established using a reverse genetics technique [5]. We have also established an avirulent recombinant H5N1 virus strain with a genetically modified haemagglutinin (HA) derived from the human

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H5N1 virus genome (Itamura & Nishimura, unpublished data), and a split-product virus vaccine was prepared using this HA-modified virus by conventional procedures [6].

Following subcutaneous administration, the inactivated vaccines induce systemic anti-viral IgG antibodies (Abs) that are highly protective against homologous virus infection but are less effective against heterologous drifted virus infection [7]. However, many studies have shown that secretory IgA (S-IgA) induced by natural infection is more cross-protective against viral infection than serum IgG induced by parenteral vaccines in humans and mice [8]. In addition, S-IgA is primarily involved in protection against infection in the upper respiratory tract, whereas serum IgG plays a role in the lower respiratory tract [9].

Previously, we demonstrated that intranasal immunisation of mice with a mixture of inactivated A or B influenza virus vaccine and cholera toxin B subunits containing a trace amount of the holotoxin (CTB*) induced both S-IgA and serum IgG [10]. We also found that S-IgA plays an important role in cross-protection against variant A and B virus infection [11,12]. In this regard, induction of S-IgA in the upper respiratory tract shows marked advantages in protection against unpredictable epidemics of influenza. Although CTB* is an effective adjuvant to produce S-IgA, it has clinical side effects, such as nasal discharge. The use of *Escherichia coli* heat-labile enterotoxin (LT), which is structurally and functionally similar to cholera toxin (CT), as an adjuvant with the nasal influenza vaccines may not be clinically safe, as intranasal influenza vaccine with LT has been linked to several cases of Bell's palsy (facial paralysis) [13]. Therefore, attempts to reduce the toxic side effects have involved the introduction of mutations into CTB [14] or by using physiological adjuvants, such as the complement component, C3d [15]. Clinically safe and more effective adjuvants are necessary for the employment of an intranasal influenza vaccine.

Recently, double-stranded RNA (dsRNA) has been reported to act as a molecular mimic associated with viral infection, because most RNA viruses produce dsRNA during their replication [16]. It has also been shown that mammalian Toll-like receptor (TLR) 3 recognises dsRNA and activates the NF-κB [17] pathway, resulting in activation of type I interferon (IFN-I), which enhances the primary antibody response against subcutaneous immunisation of soluble materials [18]. Single-stranded RNA (ssRNA) derived from influenza virus acts as a ligand for TLR 7 and induces TLR7-dependent production of IFN-α [19]. Previously, we demonstrated that the mucosal adjuvant activity of intranasal administration of synthetic dsRNA, poly (I:C) with inactivated influenza HA vaccine induces cross-protective immune responses against homologous and heterologous variant influenza virus infection [20]. In addition, chitin microparticles (CMP) were applied as an adjuvant to mice with HIV-DNA vaccine, bacterial antigen, or influenza (H1N1) HA vaccine, and resulted in both up-regulation of Th1 cytokine expression and suppression of IL-4 with reduction of allergic symptoms [21,22].

In the present study, we showed that co-administration of vaccine prepared from recombinant avirulent avian influenza virus with both poly (I:C) and CMP as mucosal adjuvants elicits protective immunity against highly pathogenic influenza virus H5N1 infection.

2. Materials and methods

2.1. Preparation of vaccine and adjuvants

A recombinant vaccine strain was produced using reverse genetics techniques. Briefly, the haemagglutinin gene of A/HongKong/156/97 (H5N1) from which the polybasic amino acid motif at the cleavage site PQRERRRKKR/G was changed to PQRETR/G, was introduced into the avirulent avian influenza A/duck/Hong Kong/836/80 (H3N1) virus as a helper strain. Using this virus (HK9-1-1, H5N1), a split-product virus vaccine was prepared in accordance with conventional methods [6,23]. The virus, grown in the allantoic cavities of 10- to 11-day embryonated hen's eggs, was concentrated, highly purified and disrupted with ether. The vaccine contained whole proteins from the virus particles, consisting mainly of HA molecules (~30% of the total protein).

Cholera toxin B subunit containing trace amounts of holotoxin (CTB*) was prepared by adding 0.1% holotoxin to cholera toxin B (Sigma, St. Louis, MO, USA). Synthetic dsRNA [poly (I:C)] was kindly provided by Toray Industries, Inc. (Kamakura, Kanagawa, Japan). CMP were prepared by sonication of dissolved purified chitin (Sigma-Aldrich, Poole, UK) in sterile, endotoxin-free phosphate-buffered saline (PBS) [21].

2.2. Immunisation protocols

All animal experiments were carried out in accordance with the Guides for Animal Experiments Performed at NIID and approved by the International Animal Care and Use Committee of the NIID. Groups of fifteen 7-week-old female BALB/c mice, or fifteen 23-week-old female B10 mice (Japan SLC Inc., Hamamatsu, Japan) were anaesthetised with diethyl ether and received a primary vaccination with 0.1–2 μg of viral protein along with 0.1–100 μg of each adjuvant or vaccine alone in a volume of 1–10 μl into each nostril. Four weeks later, they were re-immunised in the same manner with 2 μg of vaccine alone or with the same adjuvants. Two weeks after the second immunisation, five mice from each group were sacrificed to examine pre-challenge antibody responses and count the number of antibody-forming cells (AFCs), while the remaining mice were challenged intranasally with wild-type H5N1 A/Hong Kong/483/97 (HK483) virus to examine clinical signs, changes in body weight, and survival rate for 18 days.

2.3. Virus infection

HK483 virus isolated from a human patient was subcultured four times in Madin-Darby canine kidney (MDCK) cells. In accordance with a slight modification of the procedure of Yetter and co-workers [24], the mice were anaesthetised

with amobarbital sodium (0.25 ml of a 1 μg/ml solution) two weeks after the second immunisation, and 1 μl of the HK483 virus suspension was introduced into each nostril (2 μl per mouse, 100 LD_{50}). Challenge infection experiments using infectious virus were carried out under biosafety level 3 containment approved by the Guides for Animal Experiments Performed at NIID and the International Animal Care and Use Committee of the NIID.

2.4. Enzyme-linked immunosorbent assay (ELISA) for anti-H5-HA, IgA, and IgG antibodies

For measurement of anti-H5-HA antibodies, pre-challenge serum specimens were obtained from the hearts of anaesthetised mice, and nasal wash specimens were collected by washing with 1 ml of PBS containing 0.1% BSA from the nasal cavity of the excised head [10,11]. The IgA and IgG antibodies levels against recombinant HA protein of A/HongKong/483/97 (H5N1) was purified from baculovirus-infected insect cells (Katakura Industries Co., Ltd., Saitama, Japan) were determined by ELISA as described previously [11]. Standards for H5-HA-reactive IgA or IgG antibody titration were prepared as described previously [11], and expressed as the same arbitrarily units (160-unit). The antibody titres of unknown specimens were determined from the standard regression curve constructed by twofold serial dilution of the 160-unit standard for each assay.

2.5. Enzyme-linked immunospot (ELISPOT) assay

Mice were sacrificed two weeks after the second intranasal immunisation, and NALTs and spleens were removed as described previously [25]. The H5-HA protein-specific AFCs were enumerated by ELISPOT assay as described previously with minor modifications [26].

2.6. RNA isolation, cDNA synthesis, and real-time PCR

Expression of mRNAs of Toll-like receptors (TLR3, TLR4 and TLR7) in nasal-associated lymphoid tissues (NALTs) and spleens of immunised mice was examined by real-time quantitative RT-PCR. NALTs and spleens were collected from mice intranasally administered vaccine with adjuvant sequentially 6, 24, 72 h and 7 days post-immunisation. Total RNA was extracted from the NALTs and spleen of mice using an SV-Total RNA Isolation kit (Promega, Madison, WI, USA), and cDNA was synthesised using oligo-dT primer and the Omniscript Reverse Transcriptase (Qiagen, GmbH, Hilden, Germany) in accordance with the manufacturer's instructions.

Real-time quantitative RT-PCR was performed using an ABI PRISM 7900HT sequence detection system (using Ver.2.1 Software; Applied Biosystems, Foster City, CA, USA) with a QuantiTect Probe PCR kit (Qiagen), TaqMan probes, and primers (Sigma Genosys, Ishikari, Japan) designed with Primer Express (Applied Biosystems) as described previously [20]. Quantitative results were obtained using the standard curves of cycle thresholds generated from pGEM-T plasmid containing each target sequence. All data were calibrated relative to β-actin mRNA level as an endogenous internal control. Samples were considered negative if the copy number was below 10.

2.7. Pathology and immunohistochemistry of the challenged mice

Mice from either the non-immunized group or the immunised group [2 μg of vaccine combined with 10 μg of poly

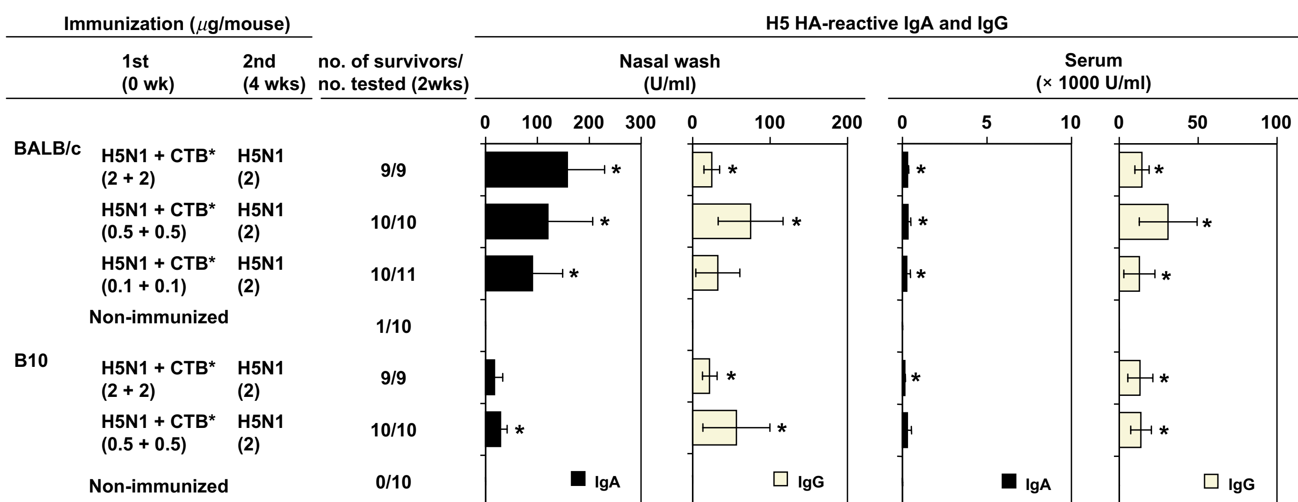


Fig. 1. Anti-H5 HA-specific IgA and IgG responses in BALB/c mice and B10 mice, and their survival rates after lethal challenge with H5N1 virus. Anti-H5 HA-specific IgA and IgG responses in BALB/c mice and B10 mice that received primary intranasal immunisation with 0.1 to 2 μg of H5 vaccine with CTB* as an adjuvant. Secondary immunisation was performed 4 weeks after primary immunisation without adjuvant. Nasal washes and serum samples were collected 2 weeks after the second immunisation. The antibody titres of the nasal wash and serum samples from five mice in each group were measured by ELISA. Mice immunised in the same way ($n = 9-11$) were infected intranasally with 100 LD_{50} of HK483 virus suspension 2 weeks after the second immunisation and their survival rates were observed. Each column represents the mean ± standard deviation (S.D.). The antibody titres were compared statistically with those of non-immunised mice with the t-test for paired observations. $*p < 0.05$.

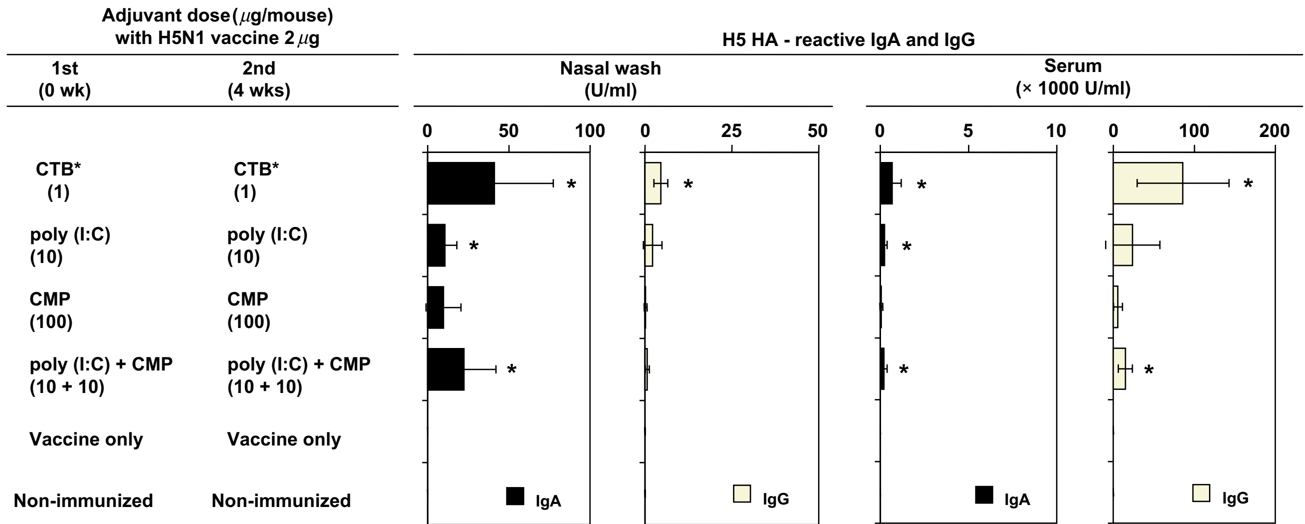


Fig. 2. Anti-H5 HA-specific antibody titre. Anti-H5 HA-specific IgA and IgG responses in BALB/c mice that received primary intranasal immunisation with 2 μg of H5 vaccine with CTB*, poly (I:C), CMP, or poly (I:C) + CMP as an adjuvant. Secondary immunisation was performed 4 weeks after primary immunisation with the same adjuvant. Nasal washes and serum samples were collected 2 weeks after the second immunisation. The antibody titres of five mice from each group were measured by ELISA. Each column represents the mean ± S.D. The antibody titres were compared statistically with those of non-immunised mice with the *t*-test for paired observations. *$p < 0.05$.

(I:C) plus 10 μg CMP] were sacrificed at 7 days post-infection for histopathological analysis. Tissues were fixed in 4% (v/v) of phosphate-buffered formalin. The tissues were then dehydrated and embedded in paraffin. Serial sections were prepared and stained with haematoxylin and eosin (H&E) solution or subjected to immunohistochemical staining with antiserum against the nucleoprotein (NP) of influenza A/PuertoRico/8/34 virus. The specificity of the anti-NP antibody and the reactivity of the antibody to H5N1 influenza virus were confirmed previously [27]. Immunohistochemical staining was performed by the avidin-streptavidin-peroxidase method using 3-3′diaminobenzidine as a substrate.

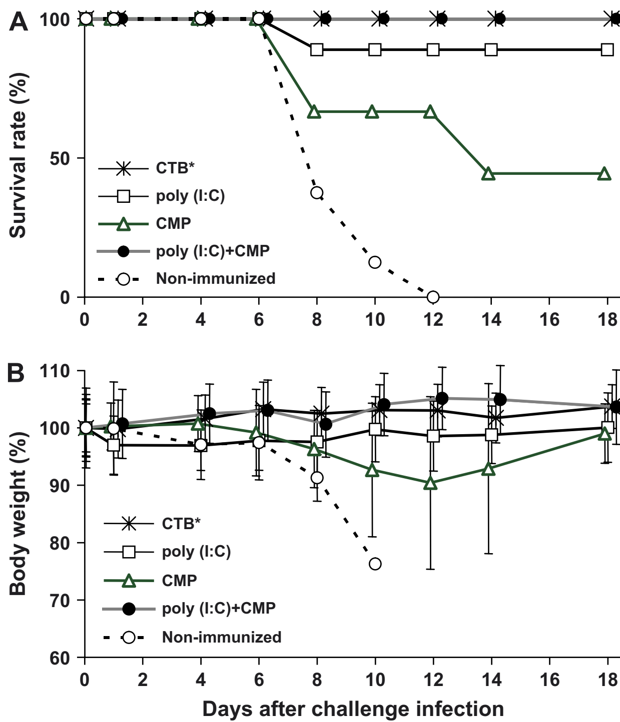


Fig. 3. Survival rates and changes in body weight of the mice after virus challenge. (A) Survival rate of the mice after HK483 challenge. Groups of mice were immunised H5 vaccine intranasally with CTB*, poly (I:C), CMP or poly (I:C) + CMP as an adjuvant and infected intranasally with 100 LD_{50} of HK483 virus suspension. (B) Changes in body weight of mice after virus challenge in the same groups as in Fig. 3A. Each point represents the mean of the relative ratio for initial body weight of 5 mice on each day.

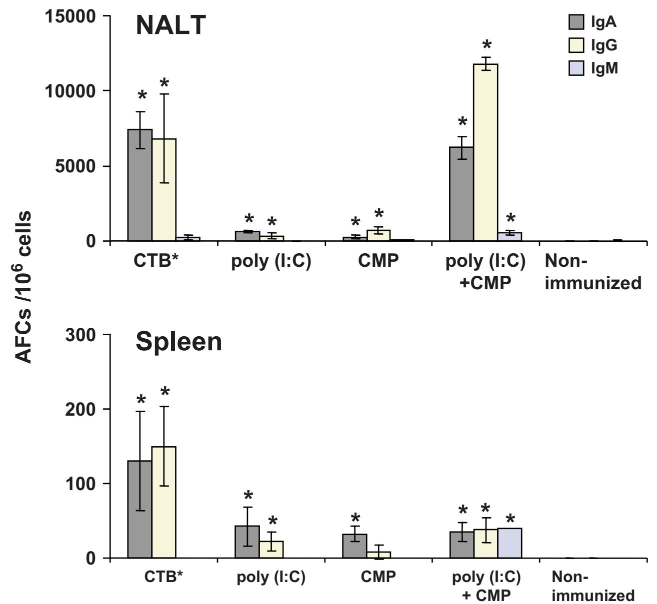
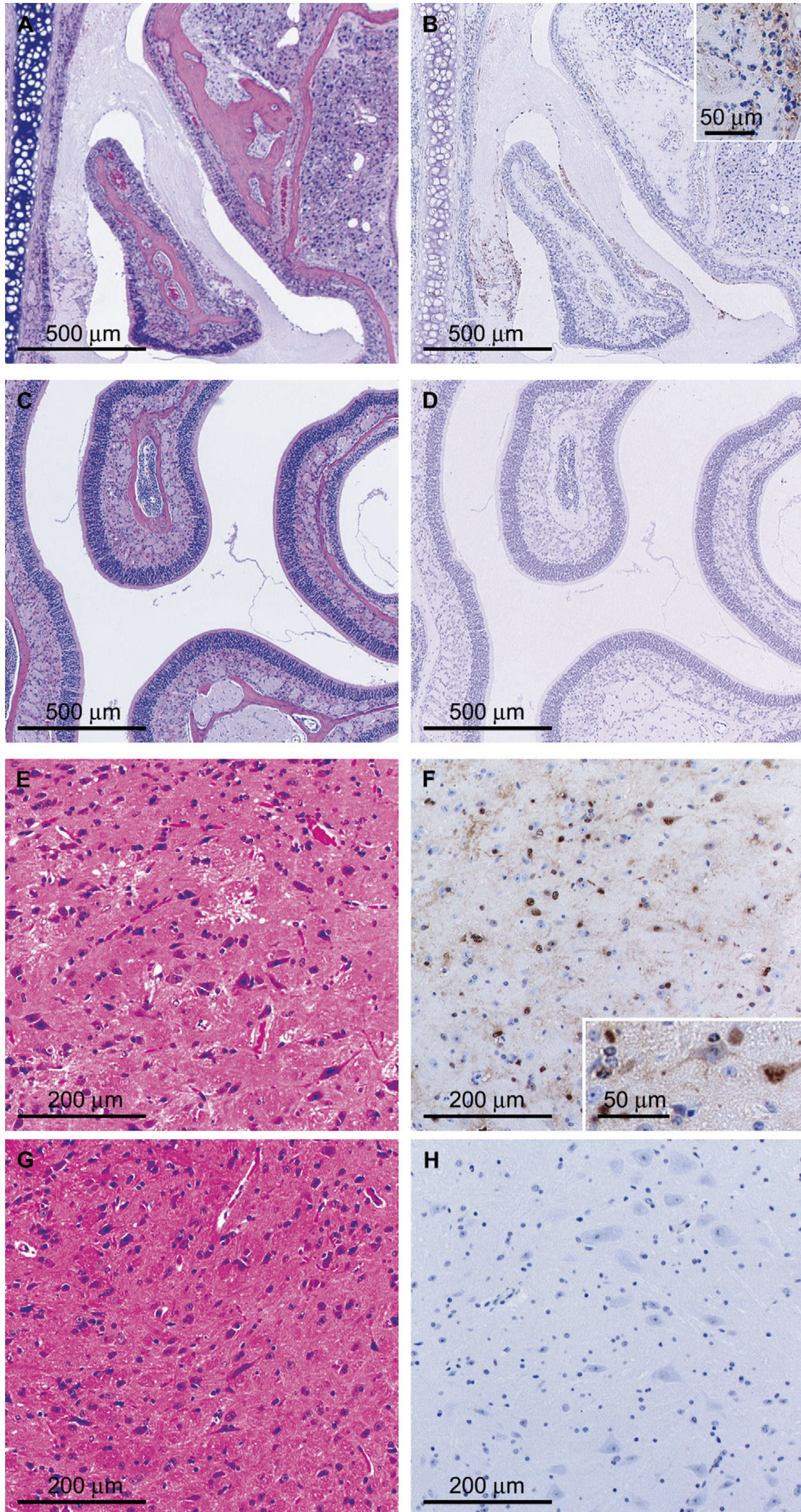


Fig. 4. ELISPOT assay of lymphocytes from the NALT and spleen of mice immunised with H5 vaccine with CTB*, poly (I:C), CMP or poly (I:C) + CMP as an adjuvant. The H5-HA protein-specific AFCs were enumerated by ELISPOT assay. The value were compared statistically with those of non-immunised mice with the *t*-test for paired observations. *$p < 0.05$.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2.8. Statistical analysis

Comparisons between experimental groups were performed by two-tailed Student's *t*-test. Values of $p < 0.05$ were considered significant unless otherwise indicated.

3. Results

3.1. Protective effect of intranasal immunisation with CTB-combined vaccine against H5N1 (HK483) infection in BALB/c and B10 mice*

Influenza virus H5N1 HA-specific IgA and IgG responses and protection against virus infection were examined in BALB/c and B10 mice immunised intranasally with two doses of HA-modified recombinant virus (H5N1) vaccine combined with CTB* (Fig. 1). BALB/c mice are high responders to the HA molecule of H1N1 influenza virus, while B10 mice are low responders. The immunisation protocol was as described in Section 2. All groups of BALB/c or B10 mice immunised with vaccine with CTB* showed HA-reactive mucosal IgA and IgG responses and serum IgG responses (Fig. 1). Vaccinated mice were also resistant against lethal challenge by H5N1 virus, while non-immunized mice were sensitive to the virus challenge (Fig. 1). Thus, intranasal administration of vaccine with CTB* was effective against H5N1 virus infection in both BALB/c and B10 mice.

3.2. Comparison of antibody responses and protective effects against H5N1 (HK483) infection in mice vaccinated with adjuvants, CTB, poly (I:C), CMP, and poly (I:C) + CMP*

To compare the adjuvant effects of poly (I:C) and CMP with CTB*, the antibody responses to H5-HA molecules were examined in BALB/c mice immunised intranasally with the vaccine and these adjuvants. The immunisation and experimental protocol was performed as described in the Materials and Methods. As we reported the effective doses of CTB*, poly (I:C) and CMP previously [20,21], we used 1 μg of CTB*, 10 μg of poly (I:C), and 10 μg or 100 μg of CMP for vaccination.

Among the adjuvants used, including CTB*, poly (I:C), CMP and poly (I:C) + CMP, CTB* induced the highest nasal IgA and serum IgG antibody titres to H5-HA, while poly (I:C) + CMP elicited weaker IgA antibody responses in the nasal wash (Fig. 2). The levels of production of IgA against H5-HA in the nasal wash in mice administered either poly (I:C) or CMP were relatively low (Fig. 2). Intranasal vaccination without adjuvant failed to induce antibody responses (Fig. 2). Subcutaneous immunisation with H5N1 vaccine without adjuvant also failed to induce antibody responses (data not shown). Thus, the use of an appropriate adjuvant enhances the efficiency of antibody production in H5N1 vaccination.

Next, we compared the protective effects of intranasal administration of the vaccine in combination with various adjuvants against lethal H5N1 infection. The time courses of survival rate and body weight loss of mice infected with H5N1 were examined for 18 days (Fig. 3A,B). After challenge infection with H5N1 (HK483) virus, non-immunised mice showed a loss of body weight with deterioration of clinical signs, such as ruffled fur, inactivity and paralysis of posterior limb. In the non-immunised group, none of the mice survived for more than 12 days after challenge infection. In contrast, none of the mice died in the groups vaccinated intranasally along with either 1 μg of CTB* or 10 μg each of poly (I:C) + CMP without any clinical signs for 18 days (Fig. 3A). Some mice in the groups vaccinated along with either poly (I:C) or CMP showed clinical signs, including a slight decrease in body weight (Fig. 3B), and died at 8 days after infection (Fig. 3A). These observations suggested that intranasal vaccination with either CTB* or concurrent use of poly (I:C) and CMP as adjuvants provided complete protection against lethal H5N1 influenza virus challenge for at least 18 days, and these protective effects seemed to be correlated with the levels of antibody production in the nasal wash and serum.

3.3. Antibody-forming cells (AFC) in the NALT and spleen of mice vaccinated intranasally with adjuvants

To examine the mechanism of intranasal vaccination, the responses of IgA AFC, IgG AFC and IgM AFC in the NALT and spleen against modified-H5-HA protein were examined in mice 2 weeks after second intranasal immunisation of vaccine with various adjuvants (Fig. 4). The numbers of IgA AFC and IgG AFC in the NALT were higher in the groups vaccinated with CTB* and poly (I:C) + CMP than in the other groups (Fig. 4). The group vaccinated with CTB* showed greater numbers of AFC cells than the other groups (Fig. 4). The number of IgM AFC in the spleen was greater in the group vaccinated with poly (I:C) + CMP than in the other groups. These results suggested that poly (I:C) + CMP as an adjuvant showed a synergistic effect on induction of AFC in the NALT as well as CTB*.

3.4. Pathological findings of the H5N1 (HK483) infected mice vaccinated intranasally with poly (I:C) + CMP

We next examined the pathological findings in non-immunised mice in comparison with those immunised intranasally with H5N1 vaccine and poly (I:C) + CMP after fatal virus challenge. Large amounts of mucous material and necrosis of the columnar epithelial cells were observed in the nasal

Fig. 5. Histopathological findings of the nasal cavity (A–D) (×40) and brain (E–H) (×100) of mice immunised intranasally with H5 vaccine with poly (I:C) + CMP (C, D, G and H) or non-immunised controls (A, B, E and F) followed by 100 LD_{50} HK483 virus infection. (×40, H&E (A, C, E, G) or ×40, anti-nucleoprotein (NP) immunostaining (B, D, F and H)). Specimens were collected 8 days after virus challenge. The insets in Fig. 5B,F are higher magnification (×200) of Fig. 5B,F. The scale bar shows 500 μm (A–D) or 200 μm (E–H).

cavity in non-immunised mice (Fig. 5A), and viral antigen was detected in the mucous material (Fig. 5B). Marked inflammatory cell infiltration with widely distributed viral antigen was also observed in the cerebrum and brainstem of non-immunised mice, consistent with viral encephalitis (Fig. 5E,F). In higher magnification, neurons are predominantly stained with anti-NP antibody (inset of Fig. 5F). Viral antigen-positive cells were much more frequent in the central nervous system than in the nasal cavity. No pathological changes and no viral antigen were detected in other organs, including the lungs and heart (data not shown). No pathological changes (Fig. 5C,G) or viral antigens (Fig. 5D,H) were detected in the nasal cavity, cerebrum or brainstem of immunised mice.

3.5. Expression of mRNAs of Toll-like receptors in the NALT and spleen of mice vaccinated intranasally with adjuvants

To determine the mechanism of action of influenza HA vaccine administered intranasally with adjuvants, we initially examined mRNA expression levels of Toll-like receptors (TLRs), including TLR3, TLR4 and TLR7, which are receptors of double-stranded RNA, lipopolysaccharide and single-stranded RNA, respectively. The levels of expression of both TLR3 and TLR7 mRNAs in the NALT of mice vaccinated with either poly (I:C) or poly (I:C) + CMP were significantly greater than those of mice vaccinated without adjuvants (7.8–12.7-fold; $p < 0.05$, Fig. 6A). TLR4 expression was significantly increased exclusively in mice vaccinated with CTB* ($p < 0.05$). These results suggested that up-regulation of TLR3 in the NALT may enhance the adjuvant effect of poly (I:C), which is consistent with a previous report [20]. In the spleen, the levels of TLR3, TLR4 and TLR7 mRNAs were slightly higher in the poly (I:C) + CMP group than the other groups (Fig. 6B). These observations suggested that the expression of TLR3 and TLR7 mRNAs in the NALT and spleen may affect expression of cytokines and production of antibodies.

4. Discussion

Until recently, direct transmission of avian influenza viruses to the human respiratory system has been considered very rare. However, it has been reported that some subtypes of avian influenza virus can replicate in the human respiratory tract after experimental infection [28]. In addition, at least 56 people died in Vietnam and Thailand between late 2003 and late 2005 (WHO), which is known as the ‘‘East Asian H5N1 epizoonotic period’’ [29]. The development of an effective vaccine offer the best strategy to protect humans against the

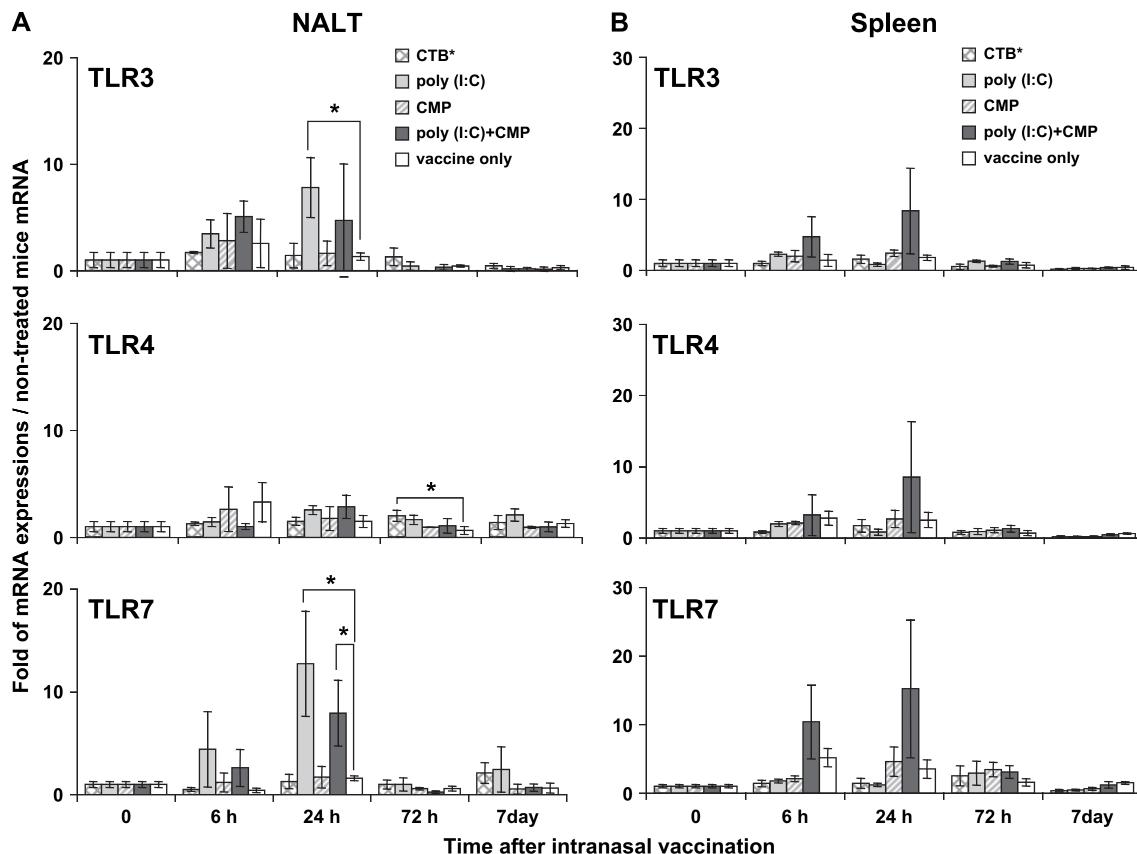

Fig. 6. Expression of TLR3, TLR4 and TLR7 mRNAs in the NALT and spleen of mice immunised intranasally with H5 vaccine with CTB*, poly (I:C), CMP or poly (I:C) + CMP at various time points (0, 6, 24, 72 h and 7 days) after vaccination. To determine the expression levels of TLR3, TLR4 or TLR7 mRNA in the NALT (A) or spleen (B), real-time quantitative RT-PCR assays were performed ($n = 3$). Comparisons between the two groups were performed with the t-test for paired observations. *$p < 0.05$.

emergence of avian influenza virus that has developed high transmissibility to humans.

As the multibasic sequence cleavage site contributes to the pathogenesis of human H5N1 infection, attenuation of the virus with modified haemagglutinin for vaccine production has been established by a plasmid-based reverse genetics system [5]. We have also prepared avirulent recombinant H5N1 (HK9-1-1) virus by reverse genetics by removal of the multibasic amino acid motif at the haemagglutinin cleavage site [23].

In 1999, Takada et al. reported that formalin-inactivated HK9-1-1 vaccine induced protective immunity in mice intranasally administered high doses (100 μg × 3 times) of vaccine [23]. As it is assumed that the supply of vaccine against the virus would be limited should the emerging virus become a pandemic, an adjuvant capable of enhancing the immunogenicity of the vaccine would conserve stocks. It has been reported that intranasal administration of inactivated vaccine with appropriate adjuvant induces higher titres of antigen-specific mucosal IgA and systemic IgG than the subcutaneous route. In addition, intranasal vaccination induces cross-reactive S-IgA antibodies that can recognize variant viruses and effectively block influenza virus infection at the mucosal membrane, which is the initial target site [11,20,21].

Bacterial toxin-derived adjuvants, such as CT and CTB have been proposed as adjuvants for mucosal vaccination [30]. Although CT is classified as a Th2-type adjuvant, it provokes nasal discharge and paralysis of facial nerves. As the nasal cavity and the forebrain have direct communication *via* the olfactory nerve, the safety of nasal administration of vaccination with adjuvant for the central nervous system needs to be considered. The safety of poly (I:C) for the central nervous system has already been confirmed by direct intracerebral injection of poly (I:C) [20]. Previously, we reported that both CMP [21] and poly (I:C) [20] are effective against H1N1 influenza vaccine by intranasal administration. However, the results of the present study demonstrated that concomitant administration of poly (I:C) + CMP was more effective than either poly (I:C) or CMP alone. In addition, poly(I:C) + CMP or poly(I:C) adjuvant is more efficient than CMP alone. Intranasal vaccination with poly (I:C) + CMP strongly enhanced nasal IgA and serum IgG against H5 HA (Fig. 2), and induced IgA- and IgG-producing AFCs in the NALT to a greater extent than vaccination with CTB* (Fig. 3). The mechanisms of the adjuvant effect of dsRNA and CMP are still unclear; however, it is known that after recognition by TLR3, RNA helicase, and retinoic acid-inducible gene I, dsRNA can activate the NF-κB pathway and production of IFN [17,31]. Early administration of IFN-α/β during an immune response markedly increases primary antibody response against soluble antigens [18]. Intranasal application of small doses (10–100 μg) of CMP has been shown to result in an elevation of Th1 cytokines, such as IL-12, IFN-γ, and TNF-α, and reduction of IL-4 production during allergen challenge [22]. It is suggested that these synergistic effects of both poly (I:C) and CMP result in marked antibody responses together with enhancement of expression of mRNAs of TLR3, TLR4, TLR7, IFN-γ, and IL-6, and confer complete protection against lethal challenge with H5N1. The use of poly (I:C) and CMP in combination enhances the cytokine responses in the spleen more effectively than the single use of poly (I:C) or CMP, and results in the enhancement of the systemic immune response. CMP can act as a carrier for poly (I:C). In addition, IFN-γ expression was enhanced in the spleen following administration of both poly (I:C) and CMP. The synergistic effects may contribute to the enhancement of mucosal adjuvant effects leading to complete protection against viral challenge.

Acknowledgements

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