PROTECTION ABILITIES OF INFLUENZA B VIRUS DNA VACCINES EXPRESSING HEMAGGLUTININ, NEURAMINIDASE, OR BOTH IN MICE

F. FANG[1*], X.Q. CAI[1†], H.Y. CHANG[1†], H.D. WANG[1], Z.D. YANG[3], Z. CHEN[1,2,3*]

[1]College of Life Science, Hunan Normal University, Changsha 410081, Hunan, P.R. China; [2]National Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, Wuhan 430071, Hubei, P.R. China; [3]Shanghai Institute of Biological Products, Shanghai 200052, P.R. China

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Summary. – Every year, a vaccination against Influenza B virus (IBV) is essential due to an antigenic variation. Development of an efficient and convenient vaccine is important for the prevention of viral infection. This study reports examination of the protective immunity in mice evoked by a single inoculation of plasmid DNA expressing hemagglutinin (HA DNA) or neuraminidase (NA DNA) of IBV. The HA DNA or NA DNA was injected intramuscularly into BALB/c mice separately or as a mixture. The injection of plasmid was followed by an electroporation close to the site of puncture. Four weeks later, the immunized mice were challenged with a lethal dose of IBV. The protective abilities of DNA vaccines were evaluated by the detection of specific antibodies in serum, survival rate, virus titer in lungs, and change of body weight. We found that a single dose of HA DNA or NA DNA induced the formation of specific antibodies and conferred effective protection against the lethal challenge of IBV. However, the combined vaccine HA DNA and NA DNA enhanced the protective ability of immunized mice. The obtained results suggested that immunization with single dose of HA DNA, NA DNA or with combination of both could be an efficient method for preventing IBV infection.

Key words: DNA vaccine; Influenza B virus; hemagglutinin; neuraminidase

Introduction

Influenza A and B viruses are responsible for considerable morbidity and mortality every year. Though IBV is less common and severe than Influenza A virus (IAV), it causes local outbreaks and infection rate can be high, particularly in younger people (Nicholson *et al*., 2003). In addition, a severe illness requiring hospitalization is seen frequently (Lin *et al*., 2006; Murphy and Webster, 1996).

IBV undergoes relatively slow antigenic variation in comparison with IAV and to date has only one recognized subtype (Nicholson *et al*., 2003). Nevertheless, there are two antigenically distinct lineages continually existing in humans, represented by the B/Yamagata/16/88 and B/Victoria/2/87 viruses (Mizuta *et al*., 2004; Shaw *et al*., 2002; Rota *et al*., 1990). Though IBV tends to be more stable than IAV, it undergoes changes too. Apart from antigenic drift, an insertion and/or deletion in the HA and NA genes together with reassortment have been shown to be the sources of antigenic diversity and evolution of IBV (Hatakeyama *et al*., 2007; Matsuzaki *et al*., 2004; Xu *et al*., 2004; McCullers *et al*., 1999). The isolation of IBV from seals in 1999 revealed that the virus is not restricted only to human beings (Osterhaus *et al*., 2000). Consequently, it raises concerns

†Both authors contributed equally to the work.
*Corresponding authors. E-mail: fangfangc@263.net; chenze2005@hotmail.com; fax: +86-7318872781.

Abbreviations: HA = hemagglutinin; HA DNA = plasmid DNA expressing hemagglutinin; IAV = Influenza A virus; IBV = Influenza B virus; NA = neuraminidase; NA DNA = plasmid DNA expressing neuraminidase**;** NI = neuraminidase inhibition; p.i. = post infection

about its potential to emerge as a virus with new antigenic properties.

Currently, prophylaxis of IBV infection is conducted by the injection of trivalent inactivated vaccine that contains two IAV strains (H1N1 and H3N2) and one IBV strain. It represents an effective measure to prevent infection, but the currently available inactivated vaccines suffer from several production limitations, especially for IBV strains (Nicholson *et al*., 2003; Hoffmann *et al*., 2002). DNA vaccine is a novel alternative to conventional one and offer many advantages not only in production but also in many other aspects (Liu, 2003). DNA-based influenza vaccines focused on IAV have been extensively investigated (Chen *et al*., 2000). Initially, the protective abilities of viral genes including HA, NA, NB, and nucleoprotein (NP) of the IBV were evaluated by Chen *et al.* (2001). It proved that both HA DNA and NA DNA applied twice by the gene gun or electroporation conferred complete protection against lethal virus challenge in mice.

However, the single administration of DNA vaccine should be much simpler and more convenient. Our recent study demonstrated that NA DNA of IAV in a single dose could provide the adequate protection against lethal homologous virus challenge in mice (Chen *et al*., 2005). Yet, the protective abilities of a single dose of IBV DNA vaccines remain to be evaluated.

Materials and Methods

Virus and cells. Influenza virus B/Ibaraki/2/85, a mouse-adapted virus strain, was propagated in Madin-Darby canine kidney (MDCK) cells. The cells were maintained in Eagles's minimal essential medium (MEM) supplemented with 10% calf serum.

Animals. SPF female BALB/c mice aged 6–8 weeks were purchased from Hubei Center of Experimental Animal (Wuhan, P.R. China) and randomly divided into groups with 12 animals per group.

DNA vaccines. Plasmids pCAGGSP7/HA and pCAGGSP7/NA were constructed by cloning of the PCR products of HA and NA genes from B/Ibaraki/2/85 virus strain into the plasmid expression vector pCAGGSP7 (Chen *et al*., 2001). Plasmids were amplified in *Escherichia coli* XL1-blue bacteria and purified by using plasmid purification kits (Qiagen).

Immunization by electroporation. In vivo electroporation was carried out according to Aihara and Miyazaki (1998). The mice were injected with a single dose of plasmid DNA into the quadriceps muscles under light anesthesia. After the injection, a pair of electrode needles 5 mm apart was inserted into the muscle to cover the DNA injection site and electric pulses were delivered using an generator (Electro Square Porator T830 M; BTX). Three pulses each of 100 V followed by the three pulses of the opposite polarity were performed on the each injection site at a rate of one pulse/sec. Each pulse lasted for 50 msec.

Virus challenge. Four weeks after the vaccination, the mice were challenged intranasally with 40 LD_{50} of the mouse-adapted virus (B/Ibaraki) in a volume of 20 µl. This infection caused rapid and widespread viral replication in the lungs followed by death of the control non-immunized mice within 7 days (Chen *et al*., 2001). After challenge, survival rate and individual body weight of mice were monitored within 21 days post infection (p.i.).

Collecting of specimens. Three days after the challenge, 6 mice from each group were anaesthetized and bled. The sera were collected and used for the determination of antibody levels in ELISA. Then, trachea and lungs were taken out and washed twice by injecting 2 ml of PBS containing 0.1% BSA. The bronchoalveolar wash was clarified by a centrifugation and used for the virus titration (Tamura *et al*., 1992).

HA antibody determination by ELISA. The levels of IgG antibodies against HA prepared from B/Ibaraki virus were determined by ELISA as previously described (Chen *et al*., 2001). The absorbance of samples was measured at $\lambda = 414$ and 405 nm in a Labsystems Multiskan Ascent Autoreader (model 354). From each absorbance reading a cut-off value (a mean for non-immune serum + 2 SD) was substracted.

NA antibody titration by neuraminidase inhibition (NI) assay. The NI assay of NA activity by anti-NA antibody was performed using fetuin (Sigma) as substrate according to the standard World Health Organization methods (Webster *et al*., 2002). The B/Ibaraki (10^7 EID_{50}/ml) grown in the 10-day-old chicken embryos and stored as allantoic fluid at -80°C was employed as enzyme source. The NI titer of the mouse antiserum was defined as the dilution of the serum inhibiting 50% of NA activity.

Virus titration. The bronchoalveolar wash was diluted 10-fold serially starting from a dilution of 1:10, inoculated on MDCK cells, incubated at 37°C, and examined for CPE 3 days later. The virus titer of each specimen expressed as $TCID_{50}$ was calculated by Reed-Muench method (Reed and Muench, 1938). The virus titer in each experimental group was presented as the mean ± SD of the virus titer/ml of specimen taken from 6 mice in each group.

Statistical analysis. Significance of differences between two mean values was evaluated by the Student's t-test; a difference with $p \leq 0.01$ was considered significant. For survival, the probability was calculated by using Fisher's exact test, comparing the survival rate of mice immunized with the viral-protein-expressing DNA with that of the control groups.

Results

Protection of mice immunized with a single dose of HA DNA or NA DNA against lethal virus challenge

In this experiment, 9 groups of mice were used. Four groups were immunized once with HA DNA at a dosage of 1, 5, 10, and 50 µg, respectively, and 4 groups with NA DNA with the same doses. The control group of mice was untreated. Four weeks after the immunization, all groups of mice were challenged by the intranasal administration of a lethal dose (40 LD_{50}) of the B/Ibaraki virus. To evaluate the ability of each dose of HA DNA or NA DNA to protect mice against acute infection, the virus titer was measured 3 days p.i. and body weight changes and survival of mice

Table 1. Determination of antibody levels, virus titer, and survival rate after IBV challenge of mice immunized with HA DNA or NA DNA

DNA vaccine	Dosage (μg)	Antibody levels in serum[a]		Virus titer in lungs[a] ($logTCID_{50}$/ml)	Survival rate (survivors/total)
		ELISA titer[b] (2^n)	NI titer[b] (2^n)		
HA	50	12.0 ± 0.7		3.7 ± 0.6*	6/6*
	10	11.5 ± 0.7		4.5 ± 0.4*	6/6*
	5	10.5 ± 1.3		4.7 ± 0.2*	5/6*
	1	6.3 ± 1.7		5.6 ± 0.3	3/6
NA	50		7.9 ± 0.2	3.3 ± 0.6*	6/6*
	10		7.2 ± 0.3	4.5 ± 0.5*	6/6*
	5		5.6 ± 0.7	4.6 ± 0.1*	6/6*
	1		2.2 ± 0.4	5.4 ± 0.7	1/6
Control				6.7 ± 0.2	0/6

[a]Values represent mean ± SD of 6 mice in each group.
[b]The serum samples were diluted 2-fold serially and "n" represents the dilution factor.
*Significant difference in comparison with control.

were monitored within 3 weeks p.i. (Table 1). The immunized mice in comparison with control mice showed a drop in the virus titer determined in lungs. The higher were the doses of DNA vaccine used, the lower were the virus titers. The survival rates of the mice immunized by HA DNA with the doses used were 50, 83.3, 100, and 100%, respectively, and those immunized by NA DNA were 16.7, 100, 100, and 100%, respectively. In contrast, the challenged non-immunized mice showed high residual lung virus titers and all died within 7 days after the challenge. The body weight of mice varied according to their health condition for all examined groups (Fig. 1a,b). The body weight of mice in 100% survival groups decreased only a little (within 5–10% of the initial body weight) and resumed their normal weight within 2 weeks. The data indicated that 5 μg of HA DNA or NA DNA could confer effective protection against lethal virus challenge in the BALB/c mice.

Protection of mice immunized with HA DNA and NA DNA combined against lethal virus challenge

To investigate the protection elicited by a single dose of combination of HA DNA and NA DNA, 3 groups of mice were immunized once with a mixture of the two plasmids at the dosage of 1, 5, and 10 μg for each component, respectively. Four weeks after immunization, the mice were challenged with a lethal dose (40 LD_{50}) of the B/Ibaraki virus. The virus titers detected in lungs were significantly lower in all immunized mice compared with those in the control mice (Table 2). The combined vaccine even at a dosage of 1 μg for each DNA could protect mice with 100% survival rate against lethal virus challenge. The mice in control group all died. Body weight changes reflected the vaccination effects (Fig. 1c). The data indicated that combined vaccine HA DNA and NA DNA containing only

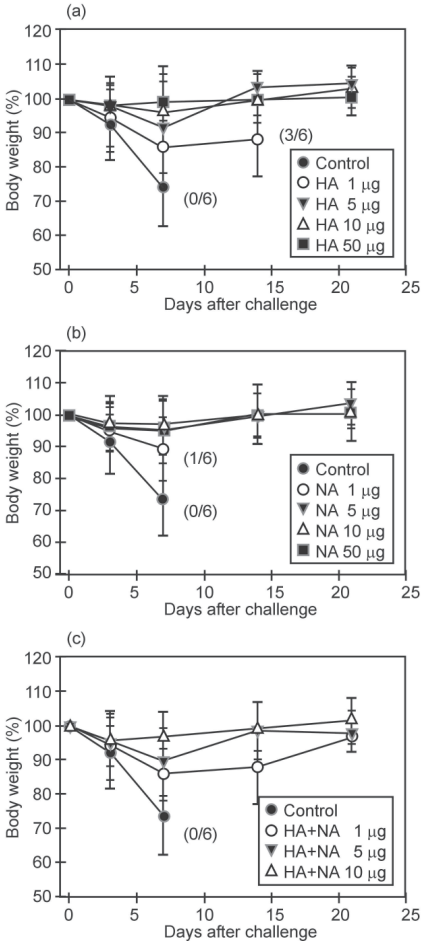

Fig. 1

Changes of body weight after virus challenge in mice

Immunization with a single dose of HA DNA (a), NA DNA (b), and combination of both (c). Values represent mean ± SD. In the group of control mice none survived (0/6) and in the group of mice immunized with 1 μg of HA DNA or NA DNA survived 3 mice (3/6) or 1 mouse (1/6), respectively.

Table 2. Determination of antibody levels, virus titer, and survival rate after IBV challenge of mice immunized with the combination of HA DNA and NA DNA

DNA vaccine	Dosage (μg)	Antibody levels in serum[a]		Virus titer in lungs[a] ($logTCID_{50}$/ml)	Survival rate (survivors/total)
		ELISA titer[b] (2^n)	NI titer[b] (2^n)		
HA + NA combined	10	10.6 ± 1.5	7.6 ± 0.3	2.9 ± 0.3*	6/6*
	5	10.3 ± 0.6	5.8 ± 0.4	3.6 ± 0.1*	6/6*
	1	6.0 ± 2.6	2.5 ± 0.2	4.0 ± 0.2*	6/6*
Control				6.7 ± 0.2	0/6

[a]Values represent mean ± SD of 6 mice in each group.
[b]The serum samples were diluted 2-fold serially and "n" represents the dilution factor.
*Significant difference in comparison with control.

1 μg of each plasmid was sufficient to confer effective protection to mice against lethal virus challenge.

Antibody response in mice immunized with a single dose of HA DNA or NA DNA or combined

Three days after the challenge, 6 mice from each group were bled and the sera were used for HA and NA antibody detection by ELISA and NI assay, respectively (Table 1 and 2). In mice injected either with HA DNA and NA DNA alone or with combination of both, the antibody levels increased along with the increased dosage of vaccine. The HA and NA antibodies in mice induced by a mixture of HA DNA and NA DNAs could reach the same levels as those induced by the same amount of HA DNA or NA DNA alone that suggested that the antigenic competition was not predominant in the combined DNA vaccine.

Discussion

In this study, we presented the protective effect of a single administration of plasmids expressing HA and NA genes alone or combined against lethal challenge with IBV in mice. We found that a single dose of HA DNA, NA DNA or combined, could confer effective protection against lethal homologous IBV challenge.

HA and NA are the major surface glycoproteins of the influenza virus. They are the most protective antigens among all viral proteins. We have already proved that HA and NA DNAs of either IAV (A/PR8) or IBV (B/Ibaraki) could provide protection in mice against lethal challenge of homologous virus, when injected twice at a 3-week interval by the electroporation (Chen *et al*., 2001, 1998). In addition, we showed in our recent work that a single inoculation of IAV (A/PR8) NA DNA could provide efficient and long-term protection against lethal virus challenge (Chen *et al*., 2005).

In this study, we demonstrated that the mice could be effectively protected against lethal challenge by the single administration of HA DNA or NA DNA derived from IBV. Generally, two or more doses are needed for plasmid DNA to elicit effective immunity in animals (Davis, 1997; Hinkula *et al*., 1997; Leitner *et al*., 1997), but a single dose of plasmid DNA has also been reported as effective (Lima *et al*., 2003; Lodmell *et al*., 2003; Moraes *et al*., 2002). We found that combined HA DNA and NA DNA vaccine administered in a single injection was very effective and the injection dosage of the combined vaccine could be reduced. The vaccine mixture containing 1 μg of each DNA (the final amount 2 μg of DNA) is sufficient to achieve an adequate protection. It means that the combination of plasmids could enhance the protective efficacy by the functional complement of the two antigens. It is apparent that HA DNA or NA DNA played different roles in preventing viral infection based on their induced antibodies. The HA antibodies neutralize the infectivity of the virus and therefore, they are primarily responsible for the prevention of infection (Tamura *et al*., 2005). However, NA antibodies block the release of newly generated virus particles from infected cells and are responsible for reduction of viral spread. Consequently, the recovery from influenza is accelerated (Tamura *et al*., 2005). We could not differentiate what type of antibodies is more important in the prophylaxis of influenza. The antigenic competition between the antigens observed in inactivated influenza vaccines, when HA and NA were presented together on intact influenza virus particles (Johansson and Kilbourne, 1993), did not seem to play a role in our experiments as was evident from the antibody levels induced. The HA and NA antibodies in mice induced by combination of HA DNA and NA DNA could reach the same level as antibodies induced by the same amount of HA DNA or NA DNA alone. The phenomenon that plasmid DNA vaccines evade antigenic competition has been repeatedly published (Zhang *et al*., 2005; Chen *et al*., 1999; Grifantini *et al*., 1998).

Vaccination against IBV is an unavoidable task every year due to antigenic variation and co-circulation of the two viral lineages. A vaccine that is not only safe and effective, but also ready to be manufactured and administered in a simple and cost-effective manner should be developed. Many types of novel vaccines against IBV were investigated to reach this goal (Bianchi *et al*., 2005; Hoffmann *et al*., 2002). The data in this paper suggested that HA DNA and NA DNA vaccines of IBV have the potential to be employed as the single-administration vaccines. This fact may promote the development of more effective IBV vaccine.

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References

Aihara H, Miyazaki J (1998): Gene transfer into muscle by electroporation in vivo. *Nat. Biotechnol.* **16**, 867–870.

Bianchi E, Liang X, Ingallinella P, Finotto M, Chastain MA, Fan J, Fu TM, Song HC, Horton MS, Freed DC, Manger W, Wen E, Shi L, Ionescu R, Price C, Wenger M, Emini EA, Cortese R, Ciliberto G, Shiver JW, Pessi A (2005): Universal influenza B vaccine based on the maturational cleavage site of the hemagglutinin precursor. *J. Virol*. **79**, 7380–7388.

Chen JJ, Fang F, Li XZ, Chang HY, Chen Z (2005): Protection against influenza virus infection in BALB/c mice immunized with a single dose of neuraminidase-expressing DNAs by electroporation. *Vaccine* **23**, 4322–4328.

Chen Z, Kadowaki S, Hagiwara Y, Yoshikawa T, Sata T, Kurata T, Tamura. S (2001): Protection against influenza B virus infection by immunization with DNA vaccines. *Vaccine* **19**, 1446–1455.

Chen Z, Kurata T, Tamura S (2000): Identification of effective constituents of influenza vaccine by immunization with plasmid DNAs encoding viral proteins. *Jpn. J. Infect. Dis*. **53**, 219–228.

Chen Z, Natsuo K, Asanuma H, Takahashi H, Iwasaki T, Suzuki Y, Aizawa C, Kurata T, Tamura S (1999): Enhanced protection against a lethal influenza virus challenge by immunization with both hemagglutinin-and neuraminidase-expressing DNAs. *Vaccine* **17**, 653–659

Chen Z, Sahashi Y, Matsuo K, Asanuma H, Takahashi H, Iwasaki T, Suzuki Y, Aizawa C, Kurata T, Tamura S (1998): Comparison of the ability of viral protein-expressing plasmid DNAs to protect against influenza. *Vaccine* **16**, 1544–1549.

Davis HL (1997): Plasmid DNA expression systems for the purpose of immunization. *Curr. Opin. Biotechnol.* **8**, 635–646.

Grifantini R, Finco O, Bartolini E, Draghi M, Del Giudice G, Kocken C, Thomas A, Abrignani A, Grandi G (1998): Multi-plasmid DNA vaccination avoids antigenic competition and enhances immunogenicity of a poorly immunogenic plasmid. *Eur. J. Immunol*. **28**, 1225–1232.

Hatakeyama S, Sugaya N, Ito M, Yamazaki M, Ichikawa M, Kimura K, Kiso M, Shimizu H, Kawakami C, Koike K, Mitamura K, Kawaoka Y (2007): Emergence of influenza B viruses with reduced sensitivity to neuraminidase inhibitors. *JAMA* **297**, 1435–1442.

Hinkula J, Svanholm C, Schwartz S, Lundholm P, Brytting M, Engstrom G, Benthin R, Glaser H, Sutter G, Kohleisen B, Erfle V, Okuda K, Wigzell H, Wahren B (1997): Recognition of prominent viral epitopes induced by immunization with human immunodeficiency virus type 1 regulatory genes. *J. Virol.* **71**, 5528–5539.

Hoffmann E, Mahmood K, Yang CF, Webster RG, Greenberg HB, Kemble G (2002): Rescue of influenza B virus from eight plasmids. *Proc. Natl. Acad. Sci. USA* **99**, 11411–11416.

Johansson BE, Kilbourne ED (1993): Dissociation of influenza virus hemagglutinin and neuraminidase eliminates their intravirionic antigenic competition. *J. Virol.* **67**, 5721–5723.

Leitner WW, Seguin MC, Ballou WR, Seitz JP, Schultz AM, Sheehy MJ, Lyon JA (1997): Immune responses induced by intramuscular or gene gun injection of protective deoxyribonucleic acid vaccines that express the circumsporozoite protein from Plasmodium berghei malaria parasites. *J. Immunol.* **159**, 6112–6119.

Lima KM, Santos SA, Lima VM, Coelho-Castelo AA, Rodrigues JM Jr, Silva CL (2003): Single dose of a vaccine based on DNA encoding mycobacterial hsp65 protein plus TDM-loaded PLGA microspheres protects mice against a virulent strain of Mycobacterium tuberculosis. *Gene Ther.* **10**, 678–685.

Lin CH, Huang YC, Chiu CH, Huang CG, Tsao KC, Lin TY (2006): Neurologic manifestations in children with influenza B virus infection. *Pediatr. Infect. Dis. J*. **25**, 1081–1083.

Liu MA (2003): DNA vaccines: a review. *J. Intern. Med.* **253**, 402–410.

Lodmell DL, Parnell MJ, Weyhrich JT, Ewalt CL (2003): Canine rabies DNA vaccination: a single-dose intradermal injection into ear pinnae elicits elevated and persistent levels of neutralizing antibody. *Vaccine* **21**, 3998–4002.

Matsuzaki Y, Sugawara K, Takashita E, Muraki Y, Hongo S, Katsushima N, Mizuta K, Nishimura H (2004): Genetic diversity of influenza B virus: the frequent reassortment and cocirculation of the genetically distinct reassortant viruses in a community. *J. Med. Virol*. **74**, 132–140.

McCullers JA, Wang GC, He S, Webster RG (1999): Reassortment and insertion-deletion are strategies for the evolution of influenza B viruses in nature. *J. Virol*. **73**, 7343–7348.

Mizuta K, Itagaki T, Abiko C, Murata T, Takahashi T, Murayama S (2004): Epidemics of two Victoria and Yamagata influenza B lineages in Yamagata, Japan. *Epidemiol. Infect*. **132**, 721–726.

Moraes MP, Mayr GA, Mason PW, Grubman MJ (2002): Early protection against homologous challenge after a single

dose of replication-defective human adenovirus type 5 expressing capsid proteins of foot-and-mouth disease virus (FMDV) strain A24. *Vaccine* **20**, 1631–1639.

Murphy BR, Webster RG (1996): Orthomyxoviruses. In Fields BN, Knipe DM, Howley PM (Eds): *Fields Virology*. Lippincott-Raven, Philadelphia, New York, pp. 1397–1445.

Nicholson KG, Wood JM, Zambon M (2003): Influenza. *Lancet* **362**, 1733–1745.

Osterhaus AD, Rimmelzwaan GF, Martina BE, Bestebroer TM, Fouchier RA (2000): Influenza B virus in seals. *Science* **288**, 1051–1053.

Reed LJ, Muench H (1938): A simple method of estimating fifty percent endpoints. *Am. J. Hyg*. **27**, 493–497.

Rota PA, Wallis TR, Harmon MW, Rota JS, Kendal AP, Nerome K (1990): Cocirculation of two distinct evolutionary lineages of influenza type B virus since 1983. *Virology* **175**, 59–68.

Shaw MW, Xu X, Li Y, Normand S, Ueki RT, Kunimoto GY, Hall H, Klimov A, Cox NJ, Subbarao K (2002): Reappearance and global spread of variants of influenza B/Victoria/2/87 lineage viruses in the 2000–2001 and 2001–2002 seasons. *Virology* **303**, 1–8.

Tamura SI, Asanuma H, Ito Y, Hirabayashi Y, Suzuki Y, Nagamine T, Aizawa C, Kurata T, Oya A (1992): Superior cross-protective effect of nasal vaccination to subcutaneous inoculation with influenza hemagglutinin vaccine. *Eur. J. Immunol*. **22**, 477–481.

Tamura S, Tanimoto T, Kurata T (2005): Mechanisms of broad cross-protection provided by influenza virus infection and their application to vaccines. *Jpn. J. Infect. Dis*. **58**, 195–207.

Webster RG, Cox N, Stoehr A (2002): WHO Manual on Animal Influenza Diagnosis and Surveillance. www.wpro.who.int/NR/rdonlyres/EFD2B9A7-2265-4AD0-BC98-97937B4FA83C/0/manualonanimalaidiagnosisandsurveillance.(pdf)

Xu X, Lindstrom SE, Shaw MW, Smith CB, Hall HE, Mungall BA, Subbarao K, Cox NJ, Klimov A (2004): Reassortment and evolution of current human influenza A and B viruses *Virus Res*. **103**, 55–60.

Zhang F, Chen J, Fang F, Zhou Y, Wu J, Chang H, Zhang R, Wang F, Li X, Wang H, Ma G, Chen Z (2005): Maternal immunization with both hemagglutinin- and neuraminidase-expressing DNAs provides an enhanced protection against a lethal influenza virus challenge in infant and adult mice. *DNA Cell Biol*. **24**, 758–765.