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Review

Identification of Effective Constituents of Influenza Vaccine by Immunization with Plasmid DNAs Encoding Viral Proteins

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CONTENTS:

SUMMARY: The use of plasmid DNA encoding influenza viral proteins to vaccinate animals constitutes a promising approach to the development of effective subunit vaccines. This review describes the results obtained by the immunization of mice with such plasmid DNAs. (i) Both hemagglutinin (HA)- and neuraminidase (NA)-expressing DNAs for the surface glycoproteins from A/PR/8/34 (H1N1) or B/Ibaraki/2/85 virus can provide the most effective protection against influenza A-type or B-type virus infection among the various viral protein-expressing DNAs tested in BALB/c mice. (ii) A mixture of plasmid DNAs encoding HA and NA can provide more effective protection against virus challenge than plasmid DNA encoding HA or NA alone in BALB/c mice. (iii) NA-DNA can provide protection against infection not only by homologous virus but also by drift viruses. (iv) HA-DNA from A/PR/8/34 (H1N1) virus provides significant protection only in BALB/c (H-2^b) mice, whereas HA-DNA from B/Ibaraki/2/85 virus affords significant protection in BALB/c, B10 (H-2^d), and C3H (H-2^k) mice. NA-DNA from both A-type and B-type viruses provides significant protection in the three strains of mice. These results suggest that both HA and NA molecules should be used as vaccine components to provide effective protection against influenza A-type and B-type virus infection in genetically heterogeneous humans.

1. Introduction

Influenza is a highly contagious acute respiratory disease, caused by infection of the host respiratory tract by the influenza virus (1, 2). The infectious virions contain eight gene segments of negative-strand RNA coding for ten viral proteins, which are nucleoprotein (NP), three polymerase proteins (PA, PB1, PB2), two matrix proteins (M1 and M2), hemagglutinin (HA), neuraminidase (NA), and two nonstructural proteins (NS1 and NS2). The RNA segments, complexed with NP and a trimeric RNA-dependent RNA polymerase (PA, PB1, PB2), make up the viral RNP (nucleoprotein-RNA-polymerase) core (Fig. 1). The core is surrounded by a layer of matrix protein (M1), which is encircled by a viral envelope, derived from the plasma membrane of the infected cells after budding. In this viral envelope, two glycoprotein spikes, HA and NA, are embedded and the ratio of HA and NA in the virions averages 5 to 1. The envelope of type A or type B influenza viruses also contains one minor component, M2 protein or NB (B-type virus NA-like molecules) protein. NS2 is also found in the virions. On the other hand, NS1 is found in abundant quantities in influenza virus-infected cells.

Infection is initiated by the binding of HA protein to a receptor on the host cells (1-3). The HA protein is a trimeric glycoprotein embedded in the viral membrane, with each monomeric glycoprotein composed of two disulfide-linked protein subunits, designated HA1 and HA2, formed through proteolytic cleavage of a single chain precursor (HA0). HA1 is responsible for the binding of the influenza virus to the sialic acid receptor on the host cell surface. Following virus attachment, virions are endocytosed by the host cell. In the acidic environment of the endosome, the hydrophobic fusion peptide of HA2 is translocated towards the target membrane so as to mediate the fusion between the endosomal membrane and the viral membrane. The viral M2 protein mediates viral uncoating by functioning as a hydrogen ion channel for the acidification of virions within the endosome. A low pH is required for the dissociation of the M1 protein from RNP complexes. Following the release of the RNP complexes from the endosome, they migrate to the nucleus where viral transcription ensues. The initial step of influenza virus replica-

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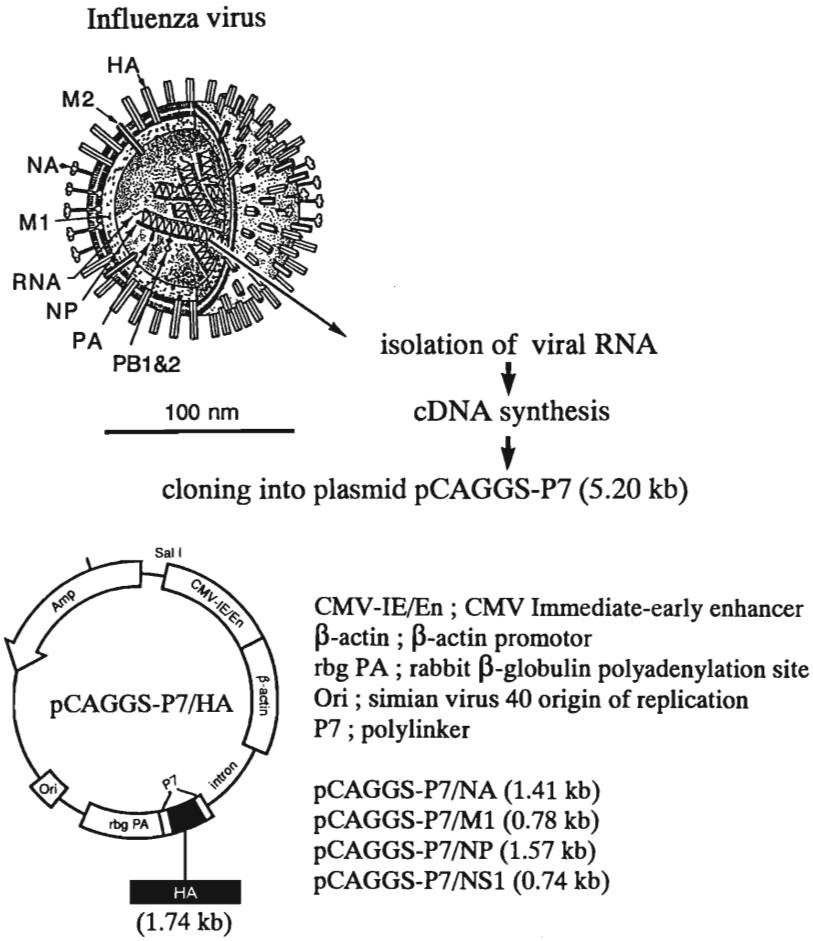

Fig. 1. Structure of influenza virus and plasmid DNA encoding HA, NA, M1, NP, or NS1 from A/PR/8/34 virus in a chicken β-actin-expressed vector (pCAGGS), constructed by Niwa, et al. (50). The diagram of a virion illustrates the main features of A-type influenza virus. The surface of the virion contains three kinds of spike proteins, the hemagglutinin (HA), neuraminidase (NA), and matrix protein (M2), embedded in a lipid bilayer derived from the host cell and covers the matrix protein (M1) that surrounds the viral core. The core is made up of the ribonucleoprotein complex which is comprised of eight single-stranded RNA segments associated with the nucleoprotein (NP) and three polymerase proteins (PA, PB1, PB2).

tion involves the transcription of its anti-sense viral RNA (vRNA) to sense messenger RNA (mRNA). Each viral gene segment then replicates. Newly assembled virus particles bud from the plasma membrane of infected cells by destroying the receptor by the action of the NA protein.

Influenza virus replicates throughout the respiratory tract and is recovered from the upper and lower respiratory tracts of people naturally or experimentally infected with the virus (2). A correlation is observed between the level of virus shedding and the magnitude of the clinical response. Since the respiratory symptoms caused by infection develop after a short incubation period, the immunity developed within several days after infection cannot prevent the onset of these symptoms. Thus, to prevent influenza, a protective immunity must be induced in advance by vaccination.

Influenza viruses are divided into types A, B, and C based on the antigenic differences between their NP and M protein antigens (1, 2). Influenza A-type viruses are further subdivided into subtype viruses (H1N1, H3N2, etc.), which are generated by marked antigenic changes in the HA and NA molecules resulting from replacement of gene segments (antigenic shift). The subtype viruses of the A-type virus cause minor antigenic changes in the HA and NA molecules (antigenic drift). Thus, influenza A-type viruses have been implicated in world-wide pandemics of respiratory diseases every year as a result of their undergoing antigenic shift and drift, while influenza B-type viruses have been implicated in local outbreaks every year as a result of undergoing antigenic drift.

To control influenza, current inactivated vaccines, which are composed of entire virions ("whole virus" vaccines), virions subjected to treatment with ether ("split-product" vaccines), or purified glycoproteins ("subunit vaccines"), are injected parenterally (2). These inactivated vaccines mainly induce serum antibodies (Abs) against HA, which directly neutralize virus infectivity. However, the influenza virus alters the antigenic properties of its HA and NA molecules, to circumvent pre-existing immunity (2, 3). Thus, the inactivated vaccines only protect against viral strains having HA molecules comprised of identical or cross-reactive epitopes. Accordingly, the protective efficacy of the vaccines is high against an epidemic of homologous virus but low against an epidemic of heterologous virus (4-6).

On the other hand, Kilbourne et al. proposed that purified NA molecules should be added to the conventional vaccines for more effective prophylaxis of influenza (7-9). Naturally or experimentally acquired Abs to NA have been reported to confer resistance to experimental or natural influenza virus infection in humans and mice (10-16). Abs to NA limit viral

replication in a multiple-cycle infection and can reduce the level of viral replication below the pathogenic threshold (17-20). Although NA, a minor component in vaccines or on the intact virion, routinely undergoes antigenic competition with immuno-dominant HA, this antigenic competition between HA and NA can be eliminated by adding NA to the vaccines as a separate purified protein (7-9, 21-25). In addition, the fact that NA evolves more slowly than HA (26) suggests that enhanced protection against infection can be achieved by immunization with an anti-influenza vaccine containing NA as an additive.

The development of a more effective vaccine remains an important goal in the control of influenza. The first step in the development of an effective influenza vaccine is to identify the viral proteins, the immune responses to which provide the highest protection against infection. Immunization with plasmid DNAs expressing viral proteins has recently proved to be a promising new approach in this respect (27). The plasmid DNA vacccine mimics natural viral infection in that antigens are produced in their native conformation and are presented in the context of major hitocompatibility complex (MHC) class I and class II molecules to elicit cytotoxic T lymphocyte (CTL) responses and other immune responses, including Ab production, respectively. Several published studies have demonstrated that plasmid DNA encoding the HA or the NP of influenza A-type viruses, administered by either a gene gun method or by intramuscular injection, elicited specific immune responses and provided protection against influenza in mice, ferrets, and chickens (28-36). NP-DNA vaccination by intramuscular injection provided cross-subtype CTL-mediated protection (28, 36). However, the most protective DNAs among influenza viral protein-expressing DNAs remain to be identified.

In addition, although influenza A-type virus DNA vaccines have been studied extensively, influenza B-type virus DNA vaccines have not. The frequency of serious B-type virus infection requiring hospitalization is about one-quarter that of influenza A-type virus infection, but B-type virus infection sometimes causes severe illness such as myocarditis and Reye's syndrome (37-46). Therefore, a study to identify the B-type viral proteins, the immune responses to which give the greatest protection against infection, remains to be conducted to prevent the disease onset.

As described above, a systematic examination for identification of the viral proteins, the immune responses to which provide the highest protection against influenza A-type or B-type virus, has not yet been made by immunization with plasmid DNAs encoding viral proteins. The objective of this review is to identify the most protective viral proteins with the aim of developing a more effective vaccine. The main part of this review is composed of our data obtained by immunization with plasmid DNAs encoding influenza A-type or B-type viral proteins.

2. Protection against influenza virus infection by immunization of mice with plasmid DNAs encoding various viral proteins

It has already been shown that Abs against HA and NA provide protection against infection by preventing infection and by reducing the level of virus replication to below the pathogeneic threshold, respectively, while Abs against NP and M1 proteins do not provide protection (1-4, 17-20). Epstein et al. have demonstrated that mice immunized with a *vaccinia* recombinant expressing the HA or NA gene of the influenza virus were protected against challenge infection by the homologous virus, while mice immunized with a *vaccinia* recombinant expressing the M1, M2, NP, NS1, NS2, or polymerase (PA, PB1 or PB2) gene, or a mixture of all eight of the latter vectors, were not protected against challenge infection (47). On the other hand, plasmid DNA encoding the NP of influenza A viruses, administered by intramuscular injection, provided cross-subtype CTL-mediated protection (28, 36). In addition, a fusion protein, combining the core antigen of a hepatitis B virus with a highly conserved portion of M2, provided protection against a lethal influenza virus challenge when administered intraperitoneally or intranasally to mice (48). Based on these results, we designed experiments to identify the most protective DNAs among influenza viral protein-expressing DNAs.

We prepared plasmid DNA encoding HA, NA, M1, NP, or NS1 from the A/PR/8/34 (PR8) (H1N1) virus in a chicken β-actin-expressing vector (pCAGGS) (Fig. 1) and compared the ability of each plasmid DNA to protect against influenza in BALB/c mice (49, 50). Each DNA was inoculated twice, 3 weeks apart, at a dose of 1 μg per mouse by particle-mediated DNA transfer to the epidermis (gene gun method). Seven days after the second immunization, the mice were challenged with homologous virus, and the ability of DNA to protect mice from influenza was evidenced by decreased lung virus titers 3 days and increased survival rate 3 weeks after the challenge infection. The results showed that mice administered HA- or NA-expressing DNA were well protected against the challenge infection (Table 1). Thus, the lung virus titer 3 days after infection in mice administered HA- or NA-expressing DNA was significantly lower than that in control mice, accompanied by a 100% survival rate 3 weeks after the challenge infection. Under these experimental conditions, the significant reduction of lung virus titers, less than $10^{4.9}$ EID_{50}/ml, correlated with long-term survival (51). The protection was accompanied by a high level of specific Ab response. The decreased lung virus titers 3 days after the challenge infection in mice, administered HA- or NA-expressing DNA may be explained by the induction of the specific Ab response rather than the induction of a CTL response, because it has been reported that the influenza virus-specific $CD8^+$ effector or memory T cells for CTL responses appear in the bronchoalveolar lavage of mice from 5 days after primary or secondary infection (52). On the other hand, mice administered M1-, NP- or NS1-DNA were not protected (Table 1), although M1- and NP-DNAs induced detectable Ab responses. It has been reported that Ab responses against M1 and NP are not involved in protection against influenza (4, 28).

In addition, we determined the level of protection against a lethal-dose influenza B virus infection in BALB/c mice immunized with plasmid DNAs encoding HA, NA, NB, and NP from the B/Ibaraki/2/85 virus (53-55). The influenza B virus NB protein is speculated to be a counterpart of the influenza A virus M2 protein which exhibits ion channel activity (2). Each DNA was administered twice, 3 weeks apart, at a dose of 1 μg per mouse by particle-mediated DNA transfer to the epidermis (gene gun method) or at a dose of 30 μg per mouse by electroporation into the muscle. Three weeks after the second immunization, the mice were challenged with a lethal dose of homologous virus. The results showed that HA- and NA-DNAs conferred protection against the lethal-dose virus challenge (Table 2). The protection was accompanied by a high level of specific Ab response. The degree of protection

Table 1. Protection against a lethal PR8 virus infection and antibody responses in mice immunized by the gene gun method with plasmid DNAs encoding various PR8 viral proteins[a]

Plasmid DNA	Protection against PR8 virus challenge		Serum antibody titers (10 days after 2nd immunization)		
	Lung virus titers (log_{10} EID_{50}/ml)	No. of survivors/ no. tested (3 weeks)	ELISA (μg/ml)	NI assay (2^n)	Immunoblotting (2^n)
HA	3.1±1.2*	5/5*	5.6±1.0		18±0
NA	3.1±0.2*	5/5		7.5±0.7	9±1
M1	6.3±0.5	0/5			21±1
NP	6.4±0.2	0/5			10±0
NS1	6.1±0.4	0/5			<1
Control	6.0±0.3	0/10	<0.1	<3	<1
Infection alone	6.1±0.2	0/10			

[a]Mice were immunized with 1 μg of plasmid DNAs encoding various viral proteins from PR8 virus on day 0 and 21 and challenged with a lethal dose of PR8 virus on day 28 (7 days after the second immunization). Lung virus titers 3 days and the survival rate 3 weeks after the challenge infection were determined as an index of protection. Serum IgG antibody titers 10 days after the second immunization were determined as an index of antibory responses. Valuse represent mean ± SD of each group of five mice. *Significant difference ($P < 0.05$) (ref. 49)

Table 2. Protection against a lethal B/Ibaraki virus infection and antibody responses in mice immunized by the gene gun method (G.G) or the electroporation method (Electro.) with plasmid DNAs encoding various B/Ibaraki viral proteins[a]

Method	Plasmid DNA	Protection against B/Ibaraki virus infection		Serum antibody titers (24 days after 2nd immunization)		
		Lung virus titers (PFU/ml, 10^n)	No. of survivors/ no. tested (3 weeks)	ELISA (2^n)	NI assay (2^n)	Immunoblotting (2^n)
G.G.	HA	2.7±0.6*	5/5*	8.6±1.2		
	NA	3.2±0.4*	5/5*		8.5±2.5	
	NB	5.8±0.1	0/5			2.5±0.7
	NP	5.7±0.2	0/5	8.5±3.1		
	Vector	5.9±0.2	0/5	<1	<3	<1
Electro.	HA	2.5±0.5*	5/5*	10.5±1.0		
	NA	2.7±0.3*	5/5*		11.8±0.5	
	NB	6.6±0.1	1/5			5.0±0
	NP	6.7±0.2	0/5	16.2±1.7		
	Vector	6.4±0.1	0/5	<1	<3	<1

[a]Mice were immunized by the gene gune method (G.G.) with 1 μg of plasmid DNAs or the electroporation method (Electro.) with 30 μg of plasmid DNAs encoding various viral proteins from B/Ibaraki virus on day 0 and 21. Three weeks after the second immunization, the mice were challenged with a lethal dose of B/Ibaraki virus. Serum samples were obtained 24 days after the second immunization. For details, see legends to Table 1. (ref. 53)

provided by gene gun immunization roughly corresponded to that provided by electroporation immunization, although the titers of anti-HA and anti-NA Abs, the production of which was induced by the gene gun method, were slightly lower than those of the Abs, the production of which was induced by the electroporation method (53). This finding suggests that even a relatively low level of anti-HA or anti-NA Ab is sufficient to prevent virus replication. On the other hand, NB- and NP-DNAs failed to provide protection against infection (Table 2), although NB- and NP-DNAs induced detectable Ab responses. Thus, Ab responses against B type virus NB and NP seem not to be involved in protection, as described for Ab responses against A type virus M1 and NP (4, 28, 49).

Our results, described above, are consistent with the results obtained using a *vaccinia* virus vector expressing influenza viral proteins (47). On the other hand, our results are different from those that showed intramuscular injection of plasmid DNA encoding NP of influenza A viruses to provide cross-subtype CTL-mediated protection (28). The discrepancy between the results can be explained by the fact that in our procedures, the mice were immunized less frequently with relatively lower doses of DNA vaccine than in other studies (27, 28, 49, 53). Less frequent immunization with relatively low doses of antigens is essential for the development of a more effective influenza vaccine. Thus, our results suggest that both HA and NA molecules can be used as vaccine components to provide effective protection against influenza A and B virus infection.

3. Protective effect of a mixture of plasmid DNAs encoding HA and NA molecules

The above results show that viral surface glycoproteins, HA and NA, are the most protective antigens among several viral proteins from A and B viruses in mice. The next step in the development of an effective vaccine against influenza A and B virus infection is to prepare a mixture of effective viral proteins rather than using a single protein.

We compared the ability of plasmid DNA encoding HA, NA, or M1 from influenza virus A/PR/8/34 (H1N1), and mixtures of these plasmid DNAs (HA+NA and HA+NA+M1) to protect against homologous virus infection in BALB/c mice (56). Mice were inoculated with each DNA, twice 3 weeks apart, at a dose of 1 μg per mouse by the gene gun method and challenged with a lethal dose of homologous virus 7 days after the second immunization. The ability of DNA to protect

mice from influenza was evidenced by decreased lung virus titers and increased survival rates. The results showed that administration of a plasmid DNA mixture of either (HA+NA) or (HA+NA+M1) provided almost complete protection in both lung virus titer and survival rate against the PR8 virus challenge (Table 3), and that this protection was accompanied by high levels of specific antibody responses to the respective components. The degree of protection afforded in these groups is significantly higher than that in mice administered either HA- or NA-expressing DNA alone, which provided significantly lower lung virus titer and a 100% suvival rate against PR8 virus challenge, or that in mice administered M1-expressing DNA, which failed to improve both lung virus titer and suvival rate. Thus, in mice immunized by the gene gun method, a mixture of plasmid DNAs encoding HA and NA provided the most effective protection against the virus challenge. The addition of M1-expressing plasmid DNA to this mixture did not enhance the degree of protection afforded.

The results described above are explained by assuming that the HA and NA molecules, derived from the respective plasmid DNAs, can be recognized independently by the host immune system and that the immune responses induced by the respective molecules are highly protective against the virus infection. This explanation may be consistent with that provided by Kilbourne et al.; that is, immunization with a mixture of purified HA and NA molecules eliminates the antigenic competition which is observed when a large amount of HA and a small amount of NA are present together on intact influenza virus particles (7-9, 21-25). Johansson and Kilbourne have also shown that no additional protection is conferred on mice immunized with M1 and NP either alone or in conjunction with other antigens (25).

4. Cross-protection against influenza virus infection provided by DNA vaccine to NA

To prevent influenza, inactivated influenza vaccines, in which HA is a major component, are injected subcutaneously. The protective efficacy of these vaccines is high against homologous virus but low against heterologous virus infection (4-6). This is because the influenza virus alters the antigenic properties of its HA molecules to escape attack by pre-existing Abs (1-3). On the other hand, Kilbourne et al. demonstrated the relatively slower antigenic evolution of NA than that of HA (26). The results reviewed in the preceding sections confirm that the NA molecule should be considered one of the most effective components for use in the development of a more protective influenza vaccine. Thus, the ability of plasmid DNAs encoding NA molecules, which alter their antigenic structure more slowly than do HA molecules, to provide cross-protection against lethal-dose heterologous virus infection remains to be determined.

We investigated whether NA-expressing DNA from different A viruses, used for the immunization of BALB/c mice, could provide cross-protection against lethal-dose heterologous influenza virus infection (57). NA-DNA, prepared from A/Guizhou/54/89 (A/Guizhou, H3N2), A/Aichi/2/68 (A/Aichi, H3N2) or A/PR/8/34 (PR8, H1N1) virus, was immunized twice, 3 weeks apart, at a dose of 1 μg per mouse by the gene gun method. Three weeks after the second immunization, mice were challenged with a lethal dose of A/Guizhou virus and the ability of each NA-DNA to protect the mice

Table 3. Protection against a lethal PR8 virus infection and antibody responses in mice immunized by the gene gun method with plasmid DNAs encoding various PR8 viral proteins[a]

Plasmid DNA	Protection against PR8 virus challenge		Serum antibody titers (10 days after 2nd immunization)		
	Lung virus titers ($\log_{10}$ EID_{50}/ml)	No. of survivors/ no. tested (3 weeks)	ELISA (μg/ml)	NI assay (2^n)	Immunoblotting (2^n)
HA	4.5±0.3*	10/10*	10.1±4.7		
NA	2.9±1.1*	10/10*		7.3±0.6	
M1	5.8±0.3	1/10			13.3±1.2
HA+NA	0.8±0.2*	10/10*	25.4±9.4	7.7±0.6	
HA+NA+M1	0.5±0.2*	10/10*	14.8±1.3	7.7±0.6	16.0±2.0
Control	6.1±0.6	0/10	<0.1	<3	<1
Infection alone	5.9±0.4	0/10			

[a]Mice were immunized twice, 3 weeks apart with 1 μg of plasmid DNAs encoding HA, NA and M1 from PR8 virus and their mixtures (HA+NA or HA+NA+M1) by the gene gun method. One week after the second immunization, mice were challenged with a lethal dose of PR8 virus. For details, see legends to Table 1. (ref. 56)

Table 4. Protection against a lethal A/Guizhou virus infection and antibody responses in mice immunized by the gene gun method with plasmid DNAs encoding the neuraminidase (NA) protein of various influenza A virus strains[a]

Plasmid DNA	Protection against Guizhou-X virus challenge		Serum antibody titers (24 days after 2nd immunization)		
	Lung virus titers (PFU/ml, 10^n)	No. of survivors/ no. tested (3 weeks)	NI assay against each virus strain		
			A/Guizhou (H3N2)	A/Aichi (H3N2)	PR8 (H1N1)
A/Guizhou NA	3.4±0.4*	10/10*	8.3±1.5	6.0±1.4	<1
A/Aichi NA	3.9±0.2*	6/10*	6.5±0.6	9.0±0.5	<1
PR8 NA	5.4±0.2	1/10	2.5±0.7	3.5±0.7	9.1±1.5
Vector	5.2±0.3	0/10	<1	<1	<1

[a]Mice were immunized by the gene gun method with 1 μg of plasmid DNAs encoding the NA proteins of various influenza virus strains on day 0 and 21. Three weeks after the second immunization, the mice were challenged with a lethal dose of A/Guizhou virus. Serum samples were obtained 24 days after the second immunization. For details, see legends to Table 1. (ref. 57)

Table 5. Protection against a lethal PR8 virus infection and antibody responses in mice immunized with plasmid DNA encoding HA, NA and NP from PR8 virus in different strains of mouse[a]

Plasmid DNA	Mouse strain (H-2)	Protection against PR8 virus challenge		Serum antibody titers (10 days after 2nd immunization)		
		Lung virus titer (log_{10} EID_{50}/ml)	No. of survivors/ no. tested (3 weeks)	Anti-HA (2^n)	Anti-NA (2^n)	Anti-NP (2^n)
HA	BALB/c (d)	4.2±0.4*	36/40*	15.2±3.2		
	C3H (k)	6.0±0.5	6/20	10.4±1.8		
	B10 (b)	6.2±0.2	6/20	4.0±1.2		
NA	BALB/c (d)	4.4±0.7*	33/40*		8.5±0.7	
	C3H (k)	5.0±0.3*	13/20*		6.5±2.1	
	B10 (b)	4.8+0.4*	13/20*		4.0±3.8	
NP	BALB/c (d)	6.1±0.2	4/40			4.2±0.4
	C3H (k)	6.0±0.1	0/20			3.8±1.3
	B10 (b)	6.1±0.2	3/20			3.6±0.9
Vector	BALB/c (d)	6.2±0.3	4/40			
	C3H (k)	5.9±0.3	3/20			
	B10 (b)	6.4±0.3	3/20			

[a]Different strains of mouse, BALB/c, B10 and C3H, were immunized with 1 μg of plasmid DNAs encoding HA, NA and NP from PR8 virus on day 0 and 21 and challenged with a lethal dose of PR8 virus on day 28 (7 days after the second immunization). For details, see legends to Table 1. (ref. 62)

from A/Guizhou virus infection was evaluated by determining the lung virus titers and the survival rate. The results showed that A/Guizhou virus NA-DNA could provide complete protection against homologous virus infection, while A/Aichi virus NA-DNA provided a significant degree of cross-protection against infection by a variant (drift) virus of the same subtype (H3N2) (Table 4). PR8 virus NA-DNA failed to provide protection against infection by a different subtype virus. The degree of cross-protection against infection correlated roughly with the titers of cross-reacting Abs. These results suggest that NA-DNA can be used as a vaccine component to provide effective protection against homologous as well as drift viruses.

5. Differences between mouse strains in development of immunity against the plasmid DNAs

The murine immune responses to various antigens and the susceptibity of mice to some pathogens are known to be controlled by H-2 or non-H-2 genes (58). We have previously reported that anti-HA Ab responses in mice immunized intranasally with inactivated influenza virus vaccines depend on the mouse strains: H-2^k, H-2^d and H-2^b strains were high, intermediate, and low responders, respectively (59). This result does not mean that strain differences in anti-HA Ab responses elicited by vaccination with HA-DNA will routinely be observed, because plasmid DNAs encoding HA stimulate the immune sytstem similarly to a viral infection but dissimilarly to exogeneous protein antigens (27, 60, 61). In this regard, strain differences in Ab responses to other viral components elicited by DNA vaccines remain to be clarified.

We compared the ability of plasmid DNAs encoding HA, NA, and NP from the A/PR/8/34 (H1N1) virus to protect against a homologous influenza A virus challenge in three different mouse strains; BALB/c (H-2^d), B10 (H-2^b) and C3H (H-2^k) (62). Mice were inoculated with each DNA twice, 3 weeks apart, at a dose of 1 μg per mouse by the gene gun method and challenged with a lethal dose of homologous virus 7 days after the second immunization. The results showed that NA-DNA could provide significant protection against infection in any mouse strain, although HA-DNA afforded significant protection only in BALB/c mice (Table 5). The levels of serum Ab titers against NA or HA molecules in BALB/c, C3H, and B10 mice were high, intermediate, and low, respectively. NP-DNA failed to provide protection in any mouse strain. These results suggest that NA-DNA can be used as an effective vaccine component to provide protection against infection in various mouse strains.

Furthermore, we compared the ability of HA- and NA-DNAs from the B/Ibaraki/2/85 virus to protect against lethal-dose influenza B-type virus infection in three different mouse strains, BALB/c, B10, and C3H (53). Each DNA was administered twice, 3 weeks apart, at a dose of 1 μg per mouse by the gene gun method or at a dose of 30 μg per mouse by electroporation into the muscle (53-55). Three weeks after the second immunization, the mice were challenged with a lethal dose of homologous virus. The results showed that both HA- and NA-DNAs provided protection against lethal-dose viral challenge in mice immunized by either the gene gun method or the electroporation method (Table 6). The degree of protection was accompanied by high levels of anti-HA and anti-NA IgG Ab responses in all of the tested mouse strains. These results suggest that both HA and NA molecules can be used as vaccine components to provide effective protection against influenza B virus infection.

The results described above show that plasmid DNA encoding HA of PR8 virus provided protection only in BALB/c mice, while plasmid DNA encoding HA of B/Ibaraki virus provided protection in three mouse strains. The discrepancy in the results for influenza A and B viruses can be explained by differences in HA-DNA (HA molecules) of both viruses. T cell epitopes on the HA and NA molecules, which are presented on antigen-presenting cells together with MHC antigens unique to each mouse strain, are different between the influenza A and B viruses (59).

6. Effectiveness of current inactivated HA vaccine supplemented with NA

The current inactivated vaccines are standardized only according to the content of the HA antigen, and HA molecules which predominate over NA molecules on an intact live

Table 6. Protection against a lethal B/Ibaraki virus infection and antibody responses in different strains of mouse immunized with plasmid DNAs encoding HA and NA from B/Ibaraki virus by the gene gun method (G.G.) or the electroporation method (Electro.)[a]

Method	Plasmid DNA	Mouse strain	Protection against B/Ibaraki virus challenge		Serum antibody titers (24 days after 2nd immunization)	
			Lung virus titer (PFU/ml, 10^n)	No. of survivors/ no. tested (3 weeks)	ELISA (2^n)	NI assay (2^n)
G.G	HA	BALB/c	3.4±0.5*	10/10*	9.0±3.5	
		C3H	3.0±0.1*	10/10*	10.0±1.6	
		B10	2.6±0.5*	10/10*	12.0±2.3	
	NA	BALB/c	3.7±0.2*	10/10*		9.3±1.5
		C3H	3.4±0.6*	10/10*		8.5±1.9
		B10	3.5±0.5*	10/10*		8.3±1.7
	Vector	BALB/c	6.0±0.2	1/10	<1	<3
		C3H	5.9±0.3	0/10	<1	<3
		B10	5.8±0.1	0/10	<1	<3
Electro.	HA	BALB/c	1.5±0.2*	10/10*	13.5±1.0	
		C3H	1.6±0.3*	10/10*	13.0±1.5	
		B10	1.5±0.2*	10/10*	15.8±2.1	
	NA	BALB/c	3.3±0.9*	10/10*		12.5±1.9
		C3H	3.6±1.5*	10/10*		13.0±1.2
		B10	3.1±0.7*	10/10*		12.8±2.2
	Vector	BALB/c	6.9±0.1	0/10	<1	<3
		C3H	6.6±0.6	2/10	<1	<3
		B10	6.9±0.2	1/10	<1	<3

[a]For details, see legends to Tables 1 and 2. (ref. 53)

influenza virus are competitive in T- and B-cell priming (21-25). Therefore, the supplementation of conventional influenza A virus vaccines with purified viral NA induces balanced and broad immune responses (7-9). Based on our previous results showing that Abs to NA molecules afford the highest degree of protection against influenza A virus infection in mice that are low responders to HA molecule administration, it seems that current inactivated vaccines supplemented with NA molecules could provide effective protection against influenza A virus infection in genetically heterogeneous humans (62). In addition, the supplementation of currently available inactivated vaccines with the purified NA of influenza A virus, as well as that of influenza B virus, could provide the most effective protection against influenza A and B virus infection in genetically heterogeneous humans (53, 56, 62).

7. Perspectives

Current trivalent inactivated vaccines, which contain components of A and B type viruses circulating in humans, are administered subcutaneously. These vaccines are shown to induce high levels of antiviral IgG Abs in serum, and have a protective effect against homologous viral infection. However, the vaccines are less effective against heterologous virus infection within the same subtype, which explains their ineffectiveness when the vaccine strain is different from the epidemic strain. On the other hand, natural influenza viral infection has been shown to be superior to current inactivated vaccines for inducing cross-protection against variant viral infections (4). The cross-protection induced by natural infection seems to be largely due to the induction of cross-reacting IgA Abs to the viral HA molecules in the respiratory tract (63). The cross-reactivity is derived from the polymeric nature of S-IgA, which generates a greater avidity for the influenza virus than does serum IgG. This fact suggests that the development of an immunization procedure to stimulate mucosal IgA production would improve the protective efficacy of the current inactivated vaccines. In this regard, intranasal immunization with vaccines has been advocated as a means of inducing IgA and systemic IgG against influenza virus (64). However, administration of the vaccine alone does not easily generate S-IgA. Therefore, we attempted to administer the vaccine together with cholera toxin (CT) or *Escherichia coli* heat-labile enterotoxin (LT) as an adjuvant (65, 66).

Intranasal immunization with current inactivated vaccines together with CTB* or LTB* (purified CTB or LTB supplemented with 0.1% of CT or 0.5% of LT) provides effective cross-protection in the upper respiratory tract against variants (drift viruses) within the subtype of the influenza A viruses or variants of the B viruses (65-69). The cross-protection in the upper respiratory tract is provided mainly by cross-reacting anti-HA IgA Abs, whereas the cross-protection in the lower respiratory tract is provided by the IgG Abs. We further investigated the vaccination conditions necessary for use of the adjuvant-combined vaccine in humans. More effective cross-protection against lethal-dose infection was provided by immunization with two doses of vaccine, than with a single dose of vaccine (51, 70). In addition, two doses of trivalent vaccines conferred cross-protection against various virus strains. From these studies, we theorized that administration of adjuvant-combined vaccine to humans could be performed effectively and safely via two successive intranasal immunizations, at 4-week intervals, with a small volume of trivalent vaccine, prepared from H1N1, H3N2, and B type virus strains (recently circulated in humans), mixed with CTB* (or LTB*) (66, 70).

In addition, as described in this review, NA molecules, as well as HA molecules, from influenza A and B viruses should be considered as some of the most effective components for use in the development of an effective influenza vaccine. This finding, together with the effectiveness of a mucosal influenza vaccine, suggests that intranasal administration of inactivated

influenza vaccines, supplemented with purified NA, can provide the most effective protection against all epidemic strains of influenza virus. Further studies are needed in order to realize the development of the most effective inactivated influenza vaccine in the near future.

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