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Immunization with influenza A NP-expressing vaccinia virus recombinant protects mice against experimental infection with human and avian influenza viruses

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Summary. Two-fold immunization of Balb/c mice with a vaccinia virus recombinant expressing the NP protein of influenza A/PR8/34 (H1N1) virus under the control of a strong synthetic promoter induced specific antibodies and protected animals against low-dose challenge by mouse-adapted heterosubtypic variants of human A/Aichi2/68 (H3N2) and avian A/Mallard/Pennsylvania/10218/84 (H5N2) influenza virus strains. The surviving immunized animals had lower antihemagglutinin antibody titers compared to non-immunized mice. There was no difference in viral titers in lungs of immunized and non-immunized animals that succumbed to the infection. In order to try to increase immune system presentation of NP-protein-derived peptides, and thereby increase their immunogenicity, we constructed another vaccinia-based NP-expressing recombinant containing a rapid proteolysis signal covalently bound to the NP protein. This sequence, derived from the mouse ornithine decarboxylase gene has been shown to increase degradation of various proteins. However, we found that when used as part of a recombinant NP, this signal neither increased its proteolytic degradation, nor was it more efficient in the induction of a protective response against influenza infection.

Introduction

Influenza infection is one of the most important public health problems of the current era [33]. Highly pathogenic avian influenza viruses of the H5N1 subtype are widespread and have become endemic in poultry in southern and southeastern

Asia [18, 26]. The threat of a worldwide epidemic caused by this or a similar virus is especially alarming. Currently, influenza vaccines capable of inducing antibodies against viral surface proteins constitute the main component of antiviral prevention instruments [14]. Such vaccines provide for a high level of resistance against the homologous influenza subtype. However, even small changes in the hemagglutinin gene will decrease vaccination efficacy. Needless to say, such changes in the influenza genome are fairly common. Thus, the creation of heterosubtypic influenza A vaccines continues to be the most pressing task in the field.

Unlike surface proteins, internal structural and nonstructural proteins of influenza virus are well conserved. They do not induce neutralizing antibodies, but stimulate the generation of cytotoxic T lymphocytes (CTL) that are capable of destroying the infected cells [33]. In particular, the major viral nucleoprotein (NP, which constitutes about 30% of virion protein content [17] continues to attract the attention of vaccine designers. This protein is very well conserved, and it does not evolve much from one subtype to another [23]. There is an abundance of data proving that this protein is capable of inducing homo- and heterosubtypic CTLs when used as a purified immunogen [32, 11] or via DNA immunization [29].

Vaccinia virus (VV) recombinants represent the conventional model for studying the immunogenicity of foreign proteins expressed therein [20]. Every influenza A gene, including NP has been expressed in VV [25]. It has been reported that VV-based influenza A-NP recombinants induce both homo- and heterosubtypic CTLs in animal models [1, 6, 21, 34].

At the same time, data on the protective capacity of NP are somewhat contradictory. Several groups did not observe sufficient protection against heterosubtypic influenza infection upon immunization with NP despite being able to demonstrate antibody and CTL generation [1–3, 27, 12, 30]. Recent data on immunization of mice with plasmid DNA encoding influenza NP have mostly shown the absence of protection against both influenza A and B [7–9]. Others have reported that immunization with NP-encoding plasmid results in a rather moderate protective effect, notably in chickens [16]. NP-expressing adenoviral recombinants did not protect against swine influenza virus, but showed improved resolution of experimental infection when used together with a HA-expressing construct [31]. At the same time, it is also possible that the immunization regimen used in at least some of these studies could be further optimized.

Therefore, we studied the protective efficacy of a vaccinia virus recombinant expressing the NP gene of influenza A/PR8 (H1N1) under the control of a strong promoter. The results of these experiments are presented in this report. We have tested the anti-NP protective response in the mouse model against challenge by both human (H3N2) and avian (H5N2) influenza viruses. Furthermore, we have attempted to increase the immunogenicity of recombinant NP by the attachment of a rapid proteolysis signal. It has been suggested that increased proteolysis of a given protein augments MHC class I presentation of the resulting peptides, thus making the protein more immunogenic [4, 35].

Materials and methods

Viruses and cells

The mouse-adapted variant of influenza A virus (family *Orthomyxoviridae*, genus *Influenzavirus A*), strain A/Aichi/2/68 (H3N2) was obtained from Dr. V. Knight (Baylor College, Texas State University, USA) and was used by us earlier [10]. The avian H5N2 influenza A virus A/Mallard/Pennsylvania/10218/84 was obtained from the virus depository of the Virology Department of St. Jude Children's Research Hospital (Memphis, TN, USA). The virus was adapted to mice by lung-to-lung passages, as described previously [24]. The influenza viruses A/WSN/33 (H1N1) and A/PR8/34 (H1N1) were obtained from the virus collection of the D.I. Ivanovsky Institute of Virology. All influenza viruses were propagated in 9-day-old embryonated chicken eggs. The virus-containing allantoic fluid was stored at 4 °C or at −70 °C. The vaccinia virus (VV) (family *Poxviridae*, subfamily *Chordopoxvirinae*, genus *Orthopoxvirus*), WR strain, was obtained from the Moscow Institute of Viral Preparations. VV-based W-NP and W-dNP recombinants expressing influenza virus NP gene were produced as described below. A VV recombinant W-NS expressing the tick-borne encephalitis virus NS1 protein gene has been described earlier [15]. The VV and its recombinants were propagated in simian kidney cells (CV1 line) and stored at −20 °C. Rat2tk$^-$ cells were used for production of VV recombinants. The canine kidney MDCK cell line was used for the titration of influenza virus from mouse lungs. All cell lines were obtained from the D.I. Ivanovsky Institute of Virology and maintained in MEM medium with 5% of bovine fetal serum.

Construction of vaccinia virus (VV) recombinants

A plasmid carrying the VV tk gene, influenza A virus NP gene, and the E. coli lacZ (pSC65-NP) was generated via conventional cloning methods. Briefly, the influenza virus A/PR8/34 (H1N1) NP gene was excised from the pCMV-PR8NPORF plasmid (kindly provided by CDC, USA) by EcoRI/SalI digestion, purified and inserted into the tk region of the pSC65 plasmid (a kind gift of Dr. B. Moss, NIH, USA), and thus placed under the control of a strong poxviral synthetic early-late promoter. VV recombinant lacZ$^+$ clones were isolated from CV1 cells simultaneously infected with VV and transfected by the pSC65-NP plasmid and purified from Rat2tk$^-$ cells in the presence of 5-brome-2′-deoxyuridine to obtain tk$^-$lacZ$^+$ clones. The presence of the NP gene in the viral recombinant was confirmed by Southern blot analysis. One of the positive clones was designated as W-NP and used thereafter.

The VV recombinant carrying degradation-prone NP protein was constructed as follows: the NP gene from pCMV-PR8NPORF was inserted into the pd1EGFP-N1 plasmid (Clontech, USA) replacing the EGFP gene. This was done in such a fashion that it resulted in the generation of an in-frame fusion between the C-end of NP protein and a region of the mouse ornithine decarboxylase (MODC) (residues 422–461) containing a PEST signal for rapid protein degradation. The construct integrity was verified by sequencing. The NP-MODC fragment from this plasmid was PCR-amplified, purified, and inserted into the pSC65 plasmid. A VV recombinant W-dNP with such a "degradation-prone" NP gene (dNP) was then generated as described above.

Virus titration

Influenza viruses were titrated in 9-day-old chick embryos, MDCK cells or 12–14 g BALB/c mice (intranasal infection, 2-week observation period) by the final dilution method. Titers were calculated by the routine Reed & Muench method and expressed as 50% embryo infection doses (EID_{50}/ml), 50% tissue culture cythopathic doses (TCD_{50}/ml), or 50% mouse lethal doses (LD_{50}/ml), respectively. VV and its recombinants were titrated in CV1 cells by the

plaque method without an agarose layer, and their titers were expressed as plaque forming units (PFU/ml).

Animal experiments

Female BALB/c mice (weight 8–10 g) were purchased from the Laboratory Animals Breeding Institution of the Russian Academy of Medical Sciences (Moscow Region, Russia). Mice were immunized twice with VV recombinants (i.p., 2-week interval between immunizations, 0.5 ml/$10^{7.0}$ PFU/mouse). Control mice were injected with PBS. Ether-anaesthetized mice were challenged intranasally 1 week after second immunization with 50 µl of PBS-diluted allantoic fluid containing 0.5–5 LD_{50} of influenza virus. Survival rates were assessed after a 2-week observation period.

Serological methods

Mouse serum samples were heat-inactivated at 56 °C for 30 min. The anti-hemagglutinin concentration was determined by the routine hemagglutinin inhibition method with chicken erythrocytes using 4 HU of the influenza viruses H3 (strain A/Aichi/2/68) and H5 (A/Mallard/Pennsylvania/10218/84). Anti-NP antibodies were quantified in ELISA using purified influenza virus A/WSN/33 as antigen [13].

Determination of NP protein in the infected cell extracts

NP protein expression was verified by Western blotting. Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), polypeptides were transferred to polyvinylidene difluoride (PVDF) 0.45-µm-pore-size membranes (Millipore, Eschborn, Germany) by semidry electroblotting in a Tris-HCl-ε-aminocaproic acid buffer system (pH ~9.8). Membranes were washed in PBS and incubated overnight at 4 °C in 10% dried milk in PBS. After washing with PBS, membranes were incubated for 2 h at room temperature in PBS containing 0.5% BSA, 0.01% Tween-20, and anti-NP mouse monoclonal antibody (clone A1, CDC, USA), PVDF membranes were then exposed to horseradish peroxidase (HRP)-conjugated secondary anti-mouse antibodies (Dako, Glostrup, Denmark), followed by visualization of positive bands by the Pierce (Rockford, Ill.) enhanced chemiluminescence (ECL) procedure using Kodak BioMax film.

Statistical methods [22]

Standard error (SE) of a percent value was determined by the formula: $SE = \sqrt{p(100 - p)/n}$, where p is percent value and n is number of animals used. Significance between two percent values (with probability 0.95):

$$t = p_1 - p_2/\sqrt{SE_1{}^2 + SE_2{}^2} > 2.0.$$

Results

CV1 cells were infected with W-NP or control W-NS recombinants using 1 PFU/cell. Cells were harvested at different time intervals and the level and specificity of NP expression was confirmed by Western blotting (Fig. 1). It can clearly be seen that the NP protein is produced in the cells infected with W-NP but not in control cells or those infected with the W-NS recombinant.

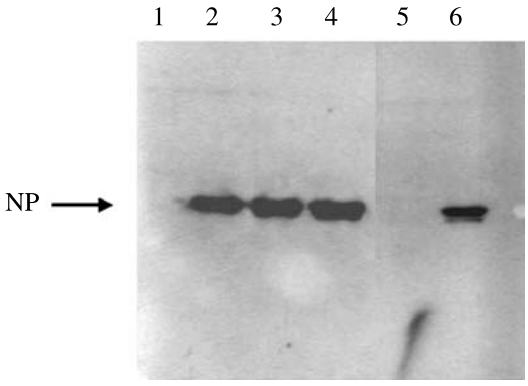

Fig. 1. Expression of influenza A virus NP protein in CV1 cells infected with vaccinia virus recombinant W-NP or W-NS (control). *1* – uninfected CV1 cells; *2* to *4* – W-NP infected CV1 cells (correspondingly: 24, 48, and 72 h post-infection); *5* – W-NS-infected CV1 cells, 48 h post-infection; *6* – purified influenza A/PR8 virus

Two-fold immunization of mice with the W-NP recombinant resulted in the generation of high-titer anti-NP specific antibodies (Table 1). Thus, we demonstrated that recombinant influenza NP protein is antigenic *in vivo*.

Table 1. Titers of anti-NP antibodies in sera of mice immunized with VV-based recombinants (determined by ELISA)

Vaccine construct	Titers of anti-NP antibodies[a]	
	Experiment 1	Experiment 2
W-NP	1:1600	1:3200
W-NS	<1:100	<1:100
None	<1:100	<1:100

[a]Serum samples of 3 mice per group were collected 1 week after the 2nd immunization prior to influenza virus infection and pooled

Table 2. Protective efficacy of VV-based recombinant W-NP against influenza virus infection *in vivo*

Immunized with	Lethal outcome in mice infected with different doses of influenza virus[a]			
	A/Aichi2/68		A/Mallard/Pennsylvania	
	1 LD_{50}	5 LD_{50}	0.5 LD_{50}	5 LD_{50}
W-NP	0/15 (0%)	3/16 (19 ± 10%)	0/19 (0%)	4/18 (22 ± 10%)
W-NS	20/37 (54% ± 8%)	30/35 (86 ± 6%)	6/38 (16 ± 6%)	30/39 (77 ± 7%)
None	8/18 (44% ± 12%)	14/17 (82 ± 9%)	6/20 (30 ± 10%)	13/20 (65 ± 11%)

[a]Numerator – number of the dead mice, denominator – number of mice per group. Overall lethality (in percent ± SE) is shown in parentheses. Immunization, challenge, and statistical methods are described in materials and methods

Immunized and control mice were challenged intranasally by mouse-adapted human A/Aichi2/68 and avian A/Mallard/Pennsylvania/10218/84 influenza viruses. The results of these experiments are summarized in Table 2. It is clear that animals immunized with W-NP exhibited a statistically significant protection against a 0.5–5 LD_{50} challenge compared to the control animals immunized with the non-specific W-NS recombinant or non-immunized mice.

Sera of surviving immunized and non-immunized animals contained antibodies against viral hemagglutinins H3 or H5 (depending on viral strain used for the challenge). The antibody titer in the sera of immunized animals was 4–8 times lower than in the sera of non-immunized surviving animals (Table 3). Thus, infection was initiated in both groups of animals. The comparatively lower level of these antibodies in the immunized mice is likely to reflect the blunting of the infection process in this group.

Infectious virus titer in the lungs of mice that succumbed to infection was not markedly different between the immunized and non-immunized groups (Table 4). A/Mallard/Pennsylvania infection resulted in a comparatively higher virus titer in lungs than infection with A/Aichi2/68.

We attempted to further enhance NP protein immunogenicity by increasing its proteolytic degradation. Our reasoning was that increased proteolytic degradation

Table 3. Titers of influenza anti-hemagglutinins in sera of mice surviving experimental influenza A infection

Immunized with	Titer of anti-hemagglutinins in sera of mice after infection with[a]			
	A/Aichi2/68		A/Mallard/Pennsylvania	
	1 LD_{50}	5 LD_{50}	0.5 LD_{50}	5 LD_{50}
W-NP	1:80	1:80	1:160	1:160
W-NS	1:640	1:640	1:640	1:1280
None	1:320	1:320	1:640	1:640

[a]Sera of 3 mice/group were collected 2 weeks after influenza virus infection and pooled

Table 4. Virus titers in lungs of mice that succumbed to experimental infection with influenza A viruses

Immunized with	Virus titers (lg TCD_{50}/ml) after infection with[a]			
	A/Aichi2/68		A/Mallard/Pennsylvania	
	1 LD_{50}	5 LD_{50}	0.5 LD_{50}	5 LD_{50}
W-NP	ND	4.5	ND	5.8
W-NS	5.2	5.0	5.8	6.2
None	4.8	4.8	6.0	5.8

[a]50% extract of lungs in PBS (lungs from 3 mice/group were pooled). Titer was measured in MDCK cells as described in materials and methods

would also increase immune system presentation of the resulting peptides. Thus, an additional VV-based recombinant, W-dNP, was constructed. The NP influenza A virus protein expressed by this recombinant bears a PEST signal attached to its carboxyl-terminus. This PEST signal, which originates from the mouse ornithine decarboxylase gene, is known to increase the proteolytic degradation of target proteins (Loetscher et al., 1991).

The degradation profiles of both recombinant proteins (W-NP and W-dNP) were assayed in the CV1 cell system. CV1 cells infected with 1 PFU/cell for 40 hours were then treated with cycloheximide (50 μg/ml) for 12 hours. After that, NP protein expression was determined by Western blotting. Recombinant NP protein was stable in the infected cells, and the PEST signal had no effect on its degradation in the vaccinia virus-based system (Fig. 2). Immunization of mice with both the W-NP and W-dNP recombinants and their subsequent challenge with A/Aichi2/68 according to the protocol described above showed

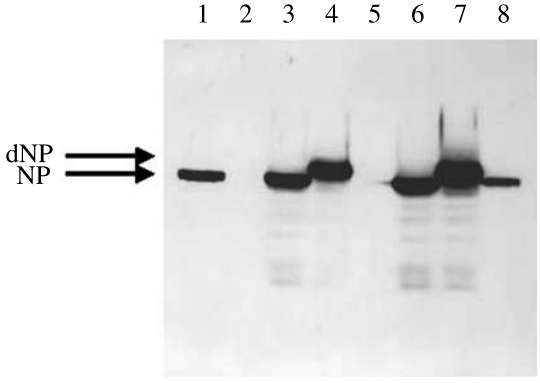

Fig. 2. Comparative stability of NP and dNP influenza virus protein during infection of CV1 cells with vaccinia virus recombinants. *1* and *8* – purified influenza A/PR8 virus (10 and 4 μl); 2 – uninfected CV1 cells; *3* to *7* – CV1 cells, infected with vaccinia recombinants and collected 40 h post-infection; *3* – W-NP + cycloheximide; *4* – W-dNP + cycloheximide; *5* – W-NS; *6* – W-NP; *7* – W-dNP. Cycloheximide (50 μg/ml) was added 28 h post-infection

Table 5. Protective efficacy of VV-based W-NP and W-dNP recombinants against influenza virus (A/Aichi2/68) infection in mice

Immunized with	Lethal outcome of influenza virus experimental infection in mice [a]	
	1 LD_{50}	10 LD_{50}
W-NP	1/19 (5 ± 5%)	13/18 (72 ± 11%)
W-dNP	0/17 (0%)	10/17 (59 ± 12%)
WR	8/12 (67 ± 14%)	11/11 (100%)
None	5/11 (45 ± 15%)	11/11 (100%)

[a] See Table 2

that these recombinant viruses possess a significant protective efficacy. However, this protection was not increased in the mice immunized with W-dNP compared to the original W-NP recombinant (Table 5).

Discussion

The data reported herein prove that immunization with a VV-based recombinant expressing influenza A virus (H1N1) NP protein is capable of generating specific antibodies in the mouse model and protecting against a challenge with heterosubtypic human H3N2 and avian H5N2 influenza A viruses.

The absence of protection in similar experiments reported by other investigators using VV-based recombinants [1–3, 27, 12, 30] can have a variety of possible explanations, including sub-optimal immunization schemes, relative weakness of the viral promoter, and test animal susceptibility to infection. Consideration must also be given to the ability of VV to suppress the presentation of NP-generated T-cell epitopes, which has also been reported earlier [28].

The VV-based recombinant used in the present study carries the NP-encoding gene under the control of the strong synthetic poxviral early-late promoter. This may explain the sufficiently high level of protective efficacy that was observed in the experiments described in this report. In addition, the two-fold scheme of immunization may also account for the increased protection level.

In our experiments we used a challenge with a low dose of infectious virus. As a result, a fraction of infected animals was found to survive in both immunized and control groups, this number being verifiably higher in the former than in the latter. Anti-hemagglutinin titers in surviving mice were measured. These titers appeared to be markedly lower in the immunized groups infected with either influenza virus strain. The difference in antibody titers can likely be explained by the blunting of the productive infection in immunized mice that survived after infection. This has subsequently resulted in a lower level of humoral immune response to influenza virus in these experimental groups.

Some of the immunized mice were not protected from the lethal effect of influenza virus. Viral titers in the lungs of the mice that succumbed to the infection were similar in immunized and non-immunized animal groups. Apparently, the level of immunity in these mice (in contrast to those that have survived) was not sufficient to prevent virus reproduction in lungs and the resulting lethal outcome.

It is thought that CTLs are the main mediators of the protection observed upon vaccination with internal viral proteins. Specific anti-NP antibodies are also generated as a result of such an immunization. As of now, the role of these antibodies in the protection process including their possible cytotoxic activity against virus-infected cells has not been particularly well-studied and needs to be elucidated further.

The protective capacities of NP protein are of the utmost importance for future influenza vaccines, since a strong immune response against this protein *in vivo*, if achieved, may provide for heterosubtypic protection. One of the main tasks in this regard is the improvement of NP immunogenicity, which could be reached via a

variety of approaches. It has been suggested that an increase in protein degradation may result in higher levels of subsequent presentation of its epitopes by the MHC class I route and thus in higher immunogenicity of recombinant protein [28, 4, 35]. Here we have used the PEST rapid proteolysis signal from the mouse ornithine decarboxylase gene [19] in order to obtain a highly degradable recombinant influenza NP protein. When this PEST signal was covalently attached to the carboxyl-terminus of recombinant NP, neither its proteolysis level nor its protective capacities were affected. Therefore, our future investigation will be aimed towards the generation of other NP recombinants with different proteolytic degradation stimuli and their subsequent testing in a variety of *in vitro* and *in vivo* model systems.

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