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Original Article

Protection against mouse and avian influenza A strains via vaccination with a combination of conserved proteins NP, M1 and NS1

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Background Experimental data accumulated over more than a decade indicate that cross-strain protection against influenza may be achieved by immunization with conserved influenza proteins. At the same time, the efficacy of immunization schemes designed along these lines and involving internal influenza proteins, mostly NP and M1, has not been sufficient.

Objective To test the immunogenicity and protective efficacy of DNA vaccination with a combination of NP, M1 and NS1 genes of influenza virus.

Methods The immunogenicity and protective efficacy of DNA vaccination with NP, M1 and NS1 was tested in mice and chickens. Mice were challenged with mouse-adapted viral strains H3N2 and H5N2 and chicken challenged with avian H5N3 virus.

Results In these settings, wild-type NS1 did not impede the cellular and humoral response to NP/M1 immunization *in vivo*. Moreover, addition of NS1-encoding plasmid to the NP/M1 immunization protocol resulted in a significantly increased protective efficacy *in vivo*.

Conclusions The addition of NS1 to an influenza immunization regimen based on conserved proteins bears promise. It is feasible that upon further genetic modification of these and additional conserved influenza proteins, providing for their higher safety, expression and immunogenicity, a recombinant vaccine based on several structural and non-structural proteins or their epitopes will offer broad anti-influenza protection in a wide range of species.

Keywords conserved proteins, cross-strain protection, influenza, vaccination.

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Introduction

A significant effort has been put towards creating a vaccine capable of inducing a broad anti-influenza response and providing protection against multiple viral strains. Different approaches have been undertaken with special focus on the induction of the cytotoxic T lymphocyte (CTL) response.[1–4]

Current inactivated vaccines are not capable of inducing strong CTL responses[5] and there is a disproportionate difference between CTL responses to various influenza epitopes.[3,6] Potentially, an influenza vaccine that is capable of generating a balanced CTL response against conserved antigens will enable a broad spectrum of coverage. Thus, a number of investigators have focused on conserved internal viral proteins NP and M1.[1,7–10] In particular, a recent study provided evidence of the significant heterosubtypic protection potential of a DNA/adenovirus combination expressing NP protein.[1] Internal proteins are conserved among influenza A strains with maximum amino acid differences of 10.8% for NP and 24.6% for M1.[11,12] Thus, it is conceivable that an immune response generated against their epitopes will provide a certain level of cross-strain protection.

Approaches that would enhance the immune response by inducing CTLs against NP have received particular attention (reviewed in Ulmer).[8] Attempts have been made to employ matrix proteins M1 and M2, but these lead to mixed results, likely due to defects in their expression and the poor immunogenicity of these proteins in their wild-type forms.[7,13–16] There are conflicting reports as to whether an immune response against NP, M1 and M2 proteins is protective against experimental influenza infection in

mice, although most of the experiments involving NP were at least partially successful.[1,7–10,13–19]

There has not been a *bona fide* attempt to investigate the possible benefits of vaccination with NS1 with the exception of a study in which plasmids carrying viral genes were tested separately.[18] NS1 was eliminated early in favour of HA, NA and NP, which in turn provided strain-specific protection based mostly on the humoral antibody response.[17–19] NS1 protein is well conserved and expressed early in the infection. A plethora of immune modulating functions is assigned to NS1, including inhibition of IFN-α and -γ.[20–24] Thus, if NS1 immunization is shown to be of value, it will likely need to be modified to enable its inclusion into a recombinant vaccine product.

CTL responses against NS1 have been detected in peripheral blood mononuclear cells (PBMC) from healthy donors. This testifies to the generation of anti-NS1 cellular immunity and to the existence of strong immune memory against this protein.[25] Alignment of NS1 gene sequences in strains from different hosts reveals that its functional domains are well conserved.[26]

Thus, in addition to NP and M1, non-structural NS1 protein is also capable of inducing a broad and long-term immune response and is a promising candidate for incorporation into a broad-spectrum influenza vaccine. In this report we present data demonstrating the protective effect of immunization with a combination of DNA plasmids encoding influenza NP, M1 and NS1. This is the first demonstration that the protective benefit achieved *in vivo* through vaccination with a combination of NP, M1 and NS1 influenza proteins is superior to the separate use of any of these immunogens or to the double combination of NP and M1.

Materials and methods

Generation of NP, M1 and NS1 expression plasmids

Expression plasmids carrying conserved influenza NP, M1 or NS1 genes (pNP, pM1 and pNS1) were constructed by insertion of the PCR-amplified full viral gene sequences into the *Eco*RI site of pCAGGS vector.[27] Viral sequences were as follows: NP from strain A/WSN/33-H1N1 (identical to A/PR/8/34-H1N1 on the amino acid level; P.O. Ilyinskii, A.G. Prilipov and A.M. Shneider, unpublished data), M1 from the same strain and NS1 from strain A/PR/8/34-H1N1 (accession numbers: V01084, L25818 and J02150). 293T cells were transfected with plasmid DNAs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's directions using either 1 or 2.7 μg of DNA per 3 cm cell culture dish.

Deletion mutants of NS1 in pCAGGS were generated by site-directed mutagenesis. The first mutant was designed to contain the deletion of amino acids 34–41 (designated as NS1del34) and the second one to contain a deletion of amino acids 34–41 and 184–188 (designated as NS1del34/184). Upon selection and sequence verification, mutant NS1 forms were tested for their expression in 293T cells.

Immunization with pNP, pM1 and pNS1 combination *in vivo*

Four micrograms of pNP, pM1 and pNS1 in 100 μl of phosphate-buffered saline (PBS) was injected intramuscularly per mouse per vaccination. For the H3N2 challenge, Balb/c mice were divided into three groups (29 animals each): control (or group 1, injected with pCAGGS), group 2 (injected with pNP/pM1/pNS1wt mixture) and group 3 (injected with pNP/pM1/pNS1del34). For the challenge with H5N2 virus, experimental groups were immunized either with: a combination of three plasmids encoding for NP, M1 or NS1 influenza proteins; with each of these plasmids separately; empty vector; or untreated control. The size of the experimental groups was 19–21 animals per group with the exception of the control group that comprised 16 animals. Mice were subjected to immunization with plasmid DNA three times with 14 day intervals in between. Animal survival, levels of antiviral CTLs and antibody generation were monitored.

CTL response *in vivo*

Six days after the third DNA vaccination, three mice from each group were killed, their splenocytes purified and stimulated ($\sim 10^8$ total, plated at 5×10^6/ml) *in vitro* by co-cultivation at a 10:1 ratio with the syngeneic feeder splenocytes infected with influenza A/PR/8/34 (H1N1) virus (taken from healthy mice, infected at MOI 20 PFU/-cell for 24 h and UV-inactivated). High levels of NP, M1 and NS1 expression in target spleen cells was demonstrated by immunoblotting with virus protein-specific antibodies (data not shown).

Splenocytes isolated from mice infected intranasally twice at 3-week intervals with a sublethal dose of influenza A/ Aichi/2/68 (H3N2) virus were used as a positive CTL control. Stimulated splenocytes were incubated for 16 days. Mouse p815 cells infected with influenza A/PR/8/34 virus (MOI 20 PFU/cell) for 24 h were used as a target and cytotoxic activity was measured by lactate dehydrogenase release (CytoTox 96 Kit; Promega, Madison, WI, USA). Target p815-infected cells (0.3×10^5/well) were mixed with twofold dilutions of stimulated effector cells starting with 3.0×10^6 cells/well and incubated for 6 h at 37°C. CTL activity as % of cell lysis was calculated by the following formula: (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100.

Humoral anti-viral response in immunized mice

The level of anti-NP and M1 antibodies was determined as follows. Serum samples of DNA-vaccinated mice were

collected on day 10 after the third DNA vaccination. Sera were assayed in a direct ELISA against whole disrupted influenza virus A/PR/8/34 adsorbed onto a plate as described earlier using a viral suspension in PBS normalized for M1 concentration of ~0.7 μg/ml (coating with 100 μl/well for 15 h at 8°C).[28] Twofold dilutions of animal sera were added to the pre-absorbed plates and virus-specific antibodies were measured employing anti-mouse IgG-horseradish peroxidase conjugate using 3,3',5,5'-tetramethylbenzidine (TMB) substrate. Radioimmunoprecipitation assay (RIPA) was performed using whole-cell lysate of Madin-Darby canine kidney (MDCK) cells infected with influenza A/WSN/33 strain (H1N1) incubated with ^{14}C yeast lysate.

Mouse influenza viruses and animal infection

The mouse-adapted variant of strain A/Aichi/2/68 (H3N2) was obtained from Dr V. Knight (Baylor College). Influenza viruses A/PR/8/34 and A/WSN/33 (H1N1) were obtained from the virus collection of Ivanovsky Institute of Virology, Russia. Viruses were propagated in 10-day-old embryonated chicken eggs. The virus-containing allantoic fluid was stored at −70°C and titrated in chicken embryo or MDCK cells. Ether-anaesthetized BALB/c mice (10–12 g) were infected intranasally with 50 μl of PBS-diluted allantoic fluid containing 10 or 100 LD_{50} of A/Aichi/2/68-MA, 9 or 10 days after the final boost. Each experimental group contained 10 animals. Protection was measured by monitoring survival and body weight, which was assessed throughout an observation period of 21 days. Severely affected mice were killed. A similar experimental set-up was used for the challenge with A/Mallard/Pennsylvania/10218/84 (H5N2). This avian influenza virus was obtained from the virus depository of the Virology Department of St. Jude Children's Research Hospital (Memphis, TN, USA) and was adapted to mice by lung-to-lung passage.[29] For both A/Aichi/2/68 and A/Mallard/Pennsylvania/10218/84 viruses, 1 LD_{50} was equal to 100–1000 $TCID_{50}$. Experimental infection was performed 9 days after the second immunization (5 LD_{50}). Lung tissues from H5N2-infected animals (two from each group) were taken at day 4 after infection for viral titre evaluation. Viral titres were measured by focus assay in MDCK cells that were grown in 24-well plates and incubated with 0.5 ml/well of 10-fold sample dilutions. After a 60-min absorption at RT, the virus inoculum was removed, cells washed and covered with 1% agarose. 50 h later, cells were fixed and incubated for 1 h with anti-influenza virus antibodies and visualized using peroxidase staining. Stained foci (PFU) were counted and titres calculated by the routine Reed & Muench method.

Determination of virus virulence in chickens

The virulence of A/Tern/South Africa/61 (H5N3) for chickens was evaluated as follows. Four-week-old chickens (Lohmann Brown, line PK-13) were infected by intramuscular injections at different dosages of virus in the range of 10–10 000 PFU/chicken. It was determined that this strain was lethal in a dose range of ~10–100 PFU/-chicken delivered intramuscularly. Infected birds lost their appetite, had ruffled feathers and manifested signs of central nervous system pathogenesis, such as inability to stand and spontaneous head twisting and succumbing to the disease within 5–8 days. The birds also shed high concentrations of virus and the virus titres in their cloak swabs reached up to 10^3–10^4 PFU/swab on day 7 post-infection.

Vaccination and experimental infection in avian model

Seventeen-day-old Lohmann Brown chickens were injected intramuscularly two times at 12-day intervals with vaccine DNA (5 μg/chicken of each DNA plasmid in 200 μl PBS per vaccination). Three groups of 10 chickens were used, these being immunized with empty vector, pNP/pM1 or pNP/pM1/pNS1 combinations. On day 9 following the second vaccination, chickens were infected intramuscularly with 10 LD_{50} of A/Tern/SA/61 virus (approximately 50–100 PFU/chicken). On day 7 following infection, blood samples and cloak swabs were prepared and virus titres were measured by virus focus assay in MDCK cells, as described above. Survival of chickens in each group was monitored daily for 15 days following infection.

Statistical methods

Standard error (SE) of a percentage value was determined by the formula: $SE = \sqrt{p(100 - p)/n}$, where p is percentage value and n is the number of animals used, similar to a previously described study.[30] The significance between two percentage values (with probability 0.95) was: $t = p_1 - p_2/\sqrt{SE_1^{2\pm}SE_2^{2}} \geq 2.0$. Animal survival was compared using log-rank test (PROC LIFETEST, SAS(R) statistical package). The differences at P-value below 0.05 were considered significant.

Results

Expression of conserved viral proteins NP, M1 and NS1 *in vitro*

Conserved wild-type influenza genes NP, M1 and NS1 were derived from H1N1 virus strains. All were shown to be efficiently expressed *in vitro* (data not shown). We also constructed two mutant variants of NS1 protein, designed to eliminate the effector regions of its immunosuppressive functions. The expression of these mutants was severely impaired compared to the robust expression of wild-type NS1 (Figure 1). Only a minor band of the NS1del34 protein was detected. No NS1-specific bands were revealed in cells transfected with the double mutant pNS1del34/184

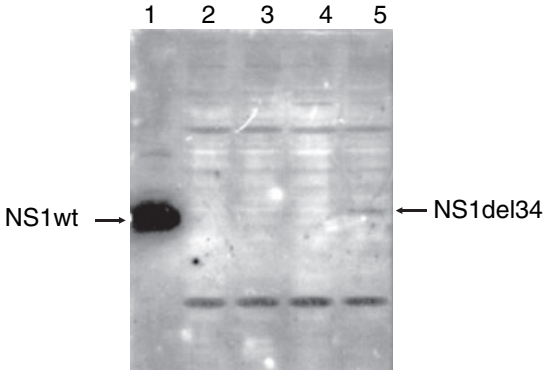

Figure 1. Expression of wild-type NS1 and its mutants *in vitro*. Total cell extract was immunoblotted with anti-NS1 polyclonal guinea-pig serum. Lane 1, NS1wt; lanes 2/3, NS1del34/184; lanes 4/5, NS1del34. Amount of protein in lane 1 is one-fifth of the amounts used in lanes 2–5. Lanes 2/3 and 4/5: 1/2.7 μg of plasmid was used for transfection.

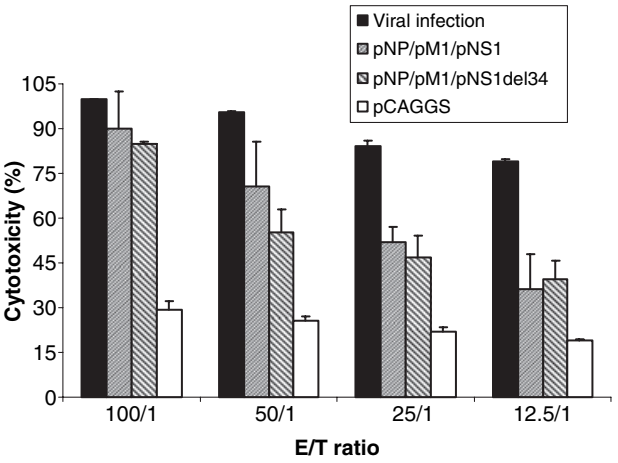

Figure 2. CTL response in animals immunized with pNS1/pNP/pM1 combinations. BALB/c mice were injected three times at 14-day intervals. Positive control – infection with sublethal dose of A/Aichi/2/68 virus; negative control – placebo immunization.

and it was not employed further. Quantitative comparison of bands produced by pNS1wt and pNS1del34 showed that expression of the mutant NS1 form was ~100 fold lower than that of NS1wt. When the proteosomal stability of NS1wt and NS1del34 was assayed using proteosomal inhibitor MG132, there was no additional stabilization and protein accumulation as seen for both mutant NS1 forms (data not shown). As it was obvious that pNS1del34 is unlikely to have any substantial activity *in vivo* it was employed throughout this study as a plasmid DNA control of immunization with pNS1.

Antiviral CTL response in immunized mice

Three groups of mice were vaccinated thrice intramuscularly with either empty vector (placebo) or with combinations of pNP/pM1/pNS1 or pNP/pM1/pNS1del34. The results of CTL measurement in animals vaccinated three times are shown in Figure 2. Significant CTL responses to influenza virus developed in all vaccinated animals. At the E/T ratio of 50:1–100:1, the CTL response in animals immunized with wild-type pNP, pM1 and pNS1 reached 70–90% of target cell lysis and was similar to CTL activity developed in native infection control (mice inoculated twice with a sublethal dose of influenza A/Aichi/2/68 virus), while the CTL response in mice vaccinated with the mixture containing pNS1del34 was somewhat lower at these high E/T ratios.

Humoral anti-viral response in immunized mice

Mouse antibody titres were determined in the sera of vaccinated animals using an ELISA against whole disrupted influenza virus (Figure 3). Unvaccinated mice infected with influenza (positive control) produced a prominent signal at serum dilutions as high as 1:128–1:256. A marked signal

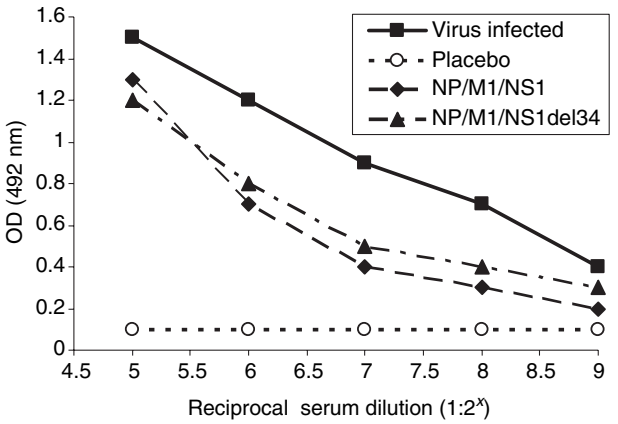

Figure 3. Antibody reactivity of sera from vaccinated animals. Twofold dilutions of sera were incubated with plated antigen (whole-disrupted influenza virus A/PR/8/34), and level of antiviral antibodies was determined as described in the text.

was also detected in mice vaccinated with pNP/pM1/pNS1 or pNP/pM1/pNS1del34 mixtures at dilutions of 1:64–1:128. Notably, the difference in the NS1 form utilized herein does not affect the antibody titre against the whole virus as the latter does not contain NS1. Sera from the pNP/pM1/pNS1-immunized group showed strong reactivity against NP and lower reactivity against M1 and NS1 in RIPA; sera from the pNP/M1/pNS1del34-immunized group showed similar reactivity to NP and M1, while the activity against NS1 was absent (data not shown).

Protective effect in experimentally infected mice

Mice vaccinated twice with both combinations of pNP, pM1 and pNS1 (differing only in the type of NS1 used)

and those in the control groups were subjected to experimental infection with influenza. All animals were challenged intranasally with the mouse-adapted variant of strain A/Aichi/2/68 (H3N2) at 10 or 100 LD_{50}. Body weight, lung pathology and overall mortality were assessed. Normal body gain was observed up to the time period following the second vaccination and preceding viral infection. These data indicate the absence of any visible toxicity of the DNA vaccine injections.

Immediately upon viral infection, a marked body weight reduction was observed in all infected groups. This reduction was fatal in placebo-immunized animals at both 10 (Figure 4) and 100 LD_{50} (data not shown), but was less dramatic in DNA vaccinated groups. The weight reduction in these groups was slower and body weight started to increase 3–4 days after virus infection, indicating recovery. The body weight gain started earlier and developed more rapidly in mice vaccinated with DNA plasmids encoding all three wild-type proteins. Less than a week after the challenge (Figure 4, day 30) the difference between pNP/pM1/pNS1 and pNP/pM1/pNS134 became statistically significant ($P \leq 0.05$).

Examination of mouse lungs was performed on day 6 following viral infection in the group that was infected with 10 LD_{50}. Two mice from each experimental group – non-vaccinated (placebo), vaccinated with either pNP/pM1/pNS1wt or pNP/pM1/pNS1del34 and uninfected – were killed, their lungs taken and photographed (Figure 5, representative results are shown). Lungs of unvaccinated mice had clear signs of fatal haemorrhagic inflammation. The inflammation in DNA-vaccinated mouse lungs was significantly less than in the lungs from the placebo control group. The most significant reduction in lung pathology was observed in mice from the group vaccinated with a combination of plasmids encoding wild-type NP, M1 and NS1. The external appearance of lungs from this animal group was similar to those of mock-infected animals (Figure 5).

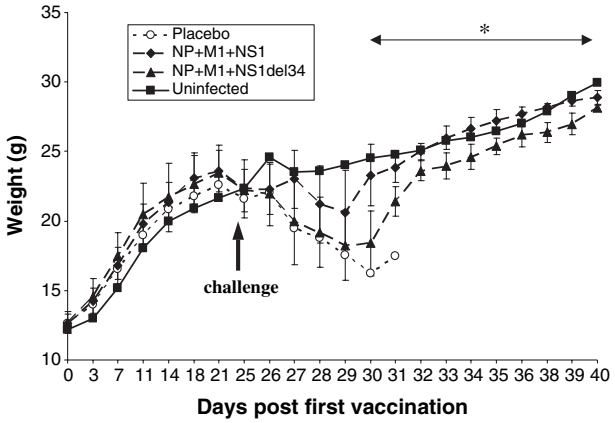

Figure 4. Body weight gain/loss in mice immunized with combinations of pNP/pM1/pNS1 and challenged with 10 LD_{50} of influenza virus. Mean weights (±SD) are shown for each group. *Time interval in which the difference between pNP/pM1/pNS1 and pNP/pM1/pNS1del34-immunized groups was statistically significant ($P < 0.05$).

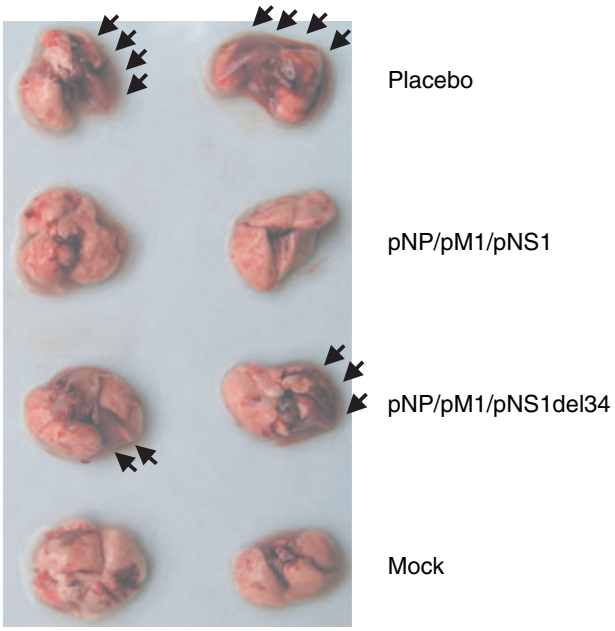

Figure 5. Lung pathology (day 6 after infection) in vaccinated and experimentally infected mice from the same experimental groups described in the legend to Figure 4. Haemorrhagic inflammation areas are shown by arrows.

Full results of animal survival following the challenge with H3N2 Aichi strain are presented in Table 1. DNA immunization with the plasmid combination of wild-type NP, M1 and NS1 proteins resulted in complete protection in the animals infected with 10 LD_{50} and showed some

Table 1. Protective efficacy of DNA immunization with conserved proteins of influenza against experimental infection with A/Aichi2/68 (H3N2) virus in mice

Immunization with	Lethal outcome of influenza virus experimental infection in mice*	
	10 LD_{50}	100 LD_{50}
pNP/pM1/pNS1	0/10	6/10
pNP/pM1/pNS1del34	4/10	8/10
Placebo	10/10	10/10

*Numerator: number of dead mice; denominator: number of mice in the group. Survival as of 21 days post-infection is shown.

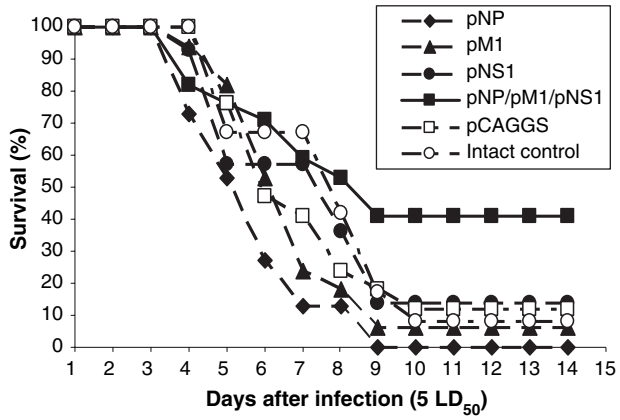

Figure 6. Survival of mice vaccinated with the combination of pNP, pM1 and pNS1 after challenge with 5 LD_{50} of H5N2 influenza virus strain A/Mallard/Pennsylvania/10218/84.

Table 2. Titres of influenza virus in lungs of mice on day 4 after infection with 5 LD_{50} of A/Mallard/Pennsylvania/10218/84

Animals immunized with	Animals tested	Geometric mean titre ± SE (log $TCID_{50}$/lung)*
pNP	4	5.93 ± 0.13
pM1	4	6.6 ± 0.18
pNS1	4	6.6 ± 0.18
pNP/pNS1/pM1	4	5.78 ± 0.16
pCAGGS	4	6.3 ± 0.18
Intact	4	6.2 ± 0.28

*Titration was done in MDCK cells (6 wells/dilution). SE calculation is described in the text.

protective effect even when 100 LD_{50} were used. Significantly less protection was provided by vaccination using a combination of NP, M1 and NS1del34. The survival difference between pNP/pM1/pNS1 and pNP/pM1/pNS1del34-immunized groups was statistically significant ($P < 0.05$) following viral challenge with 10 LD_{50}.

A separate experiment was performed in the mouse model using a similar scheme of immunization followed by challenge with a different influenza virus strain, A/Mallard/Pennsylvania/10218/84 (H5N2, of avian origin, but mouse-adapted; see Materials and methods). Six groups of Balb/c mice were inoculated either with a pNP/pM1/pNS1 combination or with each of the plasmids separately. Vaccination was performed twice and was followed by viral challenge with 5 LD_{50}. The data on animal survival are shown in Figure 6. The only group of animals that showed noticeable and statistically significant protection against 5 LD_{50} H5N2 challenge was immunized with the pNP/pM1/pNS1 combination differing significantly from pNP- and pM1-immunized groups (log-rank test, $P \leq 0.05$) as well as from pNP-, pM1- and pNS1-immunized groups combined (log-rank test, $P \leq 0.01$). This observation was further supported by the data on viral titre from infected animals (Table 2). While pNP-immunized animals also showed a decrease in viral titre (which in this group did not translate into elevated survival), it was most profoundly manifested in the group immunized with the three-plasmid combination.

Immunization and protective effect in experimentally infected chickens

We were especially interested in testing the effects of immunization with the combination of pNP/pM1/pNS1 in the avian model, which would employ another antigenically unrelated viral strain (H5N3) for the challenge. Moreover, it was imperative to test in a straightforward manner if the addition of wild-type pNS1 to pNP/pM1 combination provides an additional beneficial effect *in vivo*. Thus, we conducted a vaccination and experimental challenge experiment in the avian model using immunization either with pNP/pM1 or with pNP/pM1/pNS1.

Following the determination of the lethal infectious dose in the chicken model (see Materials and methods) we assessed the protective effect of DNA vaccination with these plasmid combinations using challenge with influenza H5N3 A/Tern/SA/61 virus. Viral titres in the infected birds were measured and their survival determined. We did not detect virus in blood samples in all of the chickens examined (3/group). Thus, there was no measurable viraemia at this stage of infection. However, virus was observed in cloak swabs taken at the same time. Significant virus titres (10^2–10^4 PFU/swab) were detected in the cloak of placebo-treated chickens. No virus was detected in the cloaks of chickens vaccinated with pNP/pM1 or pNP/pM1/pNS1 DNA combinations.

The data documenting the survival of experimentally infected chickens are shown in Figure 7. All birds vaccinated with an empty vector (placebo) died by day 8 following challenge. Marginal protection (10–20%) was observed in the group of chickens that were vaccinated with pNP/pM1 and a more prominent protective effect (40%) was observed in the group that was vaccinated with the pNP/pM1/pNS1 combination. The effect of pNP/pM1/pNS1 vaccination was statistically significant compared to placebo according to the log-rank test ($P \leq 0.02$), while vaccination with pNP/pM1 did not result in a statistically significant effect. In addition to mortality decrease, vaccination with pNP/pM1/pNS1 appeared to delay the fatal disease. Birds in this group died 1–3 days later than in the placebo group. No such effect was observed in the pNP/pM1-vaccinated group.

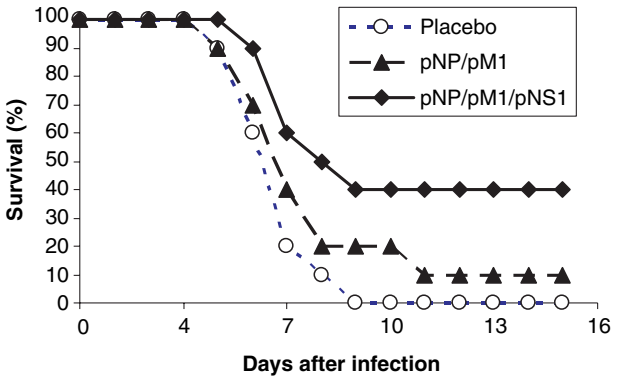

Figure 7. Survival of chickens vaccinated and experimentally infected with H5N3 influenza virus. Birds unvaccinated and vaccinated with either pNP/pM1 or pNP/pM1/pNS1 were challenged with lethal doses of H5N3 A/Tern/SA/61 avian influenza virus as described in the text.

Discussion

Many research groups including ours have repeatedly demonstrated that internal proteins of influenza virus may provide for some degree of protection against infection.[1,7,9,10,17–19,30] Moreover, immunization against NP was thought to carry the greatest promise of generating a broad-spectrum anti-influenza vaccine.[8,31] At the same time, another conserved influenza protein, NS1, has not been studied as a component of a recombinant vaccine, possibly due to the capacity of this protein to suppress multiple immune response pathways.[20–24] However, if the benefits of NS1 incorporation into an immunization regimen are demonstrated, it would be very important to generate a safe and immunologically effective NS1 variant. Therefore, we attempted to construct NS1 mutants designed to abrogate known determinants of its immunosuppressive functions.

While wild-type NS1 was efficiently expressed, none of the NS1 mutants showed strong expression *in vitro*. In fact, only one, NS1del34, was verifiably detected. The extremely low expression level of this mutant cannot be attributed to the higher degree of its proteosomal degradation as the proteosome inhibitor, MG132, did not increase the level of NS1del34 (otherwise, one could argue that mutant NS1 may be misfolded and thus rapidly degraded by proteosome-dependent mechanisms and ultimately, efficiently presented by the major histocompatibility complex class I pathway). Moreover, the region encompassing amino acids 34–41 is generally important for NS1 expression as a mutant that has only two substitutions in amino acids 38 and 41 was also not expressed *in vitro* in a proteosome-independent manner. This was not related to epitope recognition as this novel NS1 mutant was fused to peptide tags on both N- and C-termini, and antibodies to these tags were used for NS1 detection. Furthermore, we have demonstrated that the expression of this and similar NS1 mutants is markedly hindered. Apparently, this region of the NS1-encoding RNA possesses a conserved sequence that plays an important role in its stabilization and translation initiation (Ilyinskii *et al.*, unpublished data).

The immunogenicity of the plasmid combinations pNP/pM1/pNS1 and pNP/pM1/pNS1del34 was studied in the mouse model. Both of these combinations efficiently induced CTLs *in vivo*. Notably, the anti-influenza CTL response in naturally infected humans is known to develop mainly against NP, M1 and NS1.[3,25,32]

The protective capacities of vaccination with these plasmid combinations were assessed using intranasal challenge with 10 or 100 LD_{50} of mouse lung-adapted A/Aichi/2/68 (H3N2) virus. Body weight, lung pathology and overall survival were monitored. Thus it was clear that immunization with a combination of pNP/pM1/pNS1 resulted in a significant protective benefit. Such an effect was less evident for the pNP/pM1/pNS1del34-immunized group.

Due to the very low expression level ($\sim$ 100-fold less than NS1wt) of NS1del34, one may provisionally count the latter group as immunized with only pNP/pM1, but with the same overall DNA quantity as the pNP/pM1/pNS1-immunized group. If such an assumption is correct, then it is clear that the addition of NS1wt provides a clear benefit in terms of animal survival, which was statistically significant for the groups infected with 10 LD_{50} ($P < 0.05$). The results reported earlier demonstrated no protection when NS1 alone was used for immunization.[18]

Furthermore, we employed a more stringent challenge of similarly immunized mice with 5 LD_{50} of antigenically unrelated H5N2 virus that originated in birds but adapted to mice by serial passages, a process not dissimilar from the one that may result in the human adaptation of an avian pandemic strain. In this system, the only immunization regimen that was beneficial also comprised pNP/pM1/pNS1, while these plasmids when used individually provided no alleviation of disease.

It was important to test whether such a protective effect could be observed in a different host and against a very divergent influenza virus strain. Therefore, we conducted an experiment in a chicken model using antigenically unrelated H5N3 influenza virus. Two vaccine combinations were employed: pNP/pM1 and pNP/pM1/pNS1. Of these, only the latter combination has shown a protective benefit in the avian system. These results confirm that the inclusion of NS1 into the vaccination regimen provides an additional benefit against morbidity and mortality in the experimental setting.

In this experimental series we have used a hetero-subtypic challenge upon the homologous immunization with suboptimal DNA vectors. Moreover, we have deliberately used two immunizations in our protection studies (compared to

three immunizations in initial immunogenicity tests) to move away from the idealized laboratory-type regimens and towards more realistic vaccination schedules (in addition, DNA is known to be a much better prime than booster and it is unlikely that the third DNA immunization will result in a dramatic elevation of immune responses). While the NP/M1/NS1 combination has, in our view, shown some promise for cross-strain protection, this immunization regimen and vectors used herein may be significantly improved. The use of adenoviral vectors and heterologous prime-boost appears to be particularly promising.[1,33,34] It is not improbable to reach a high level of protection using exclusively immunization with conserved influenza viral proteins or their derivatives, especially if newly developed approaches are used in concert, including novel adjuvants, vectors and other immune response augmentation strategies.

Author contribution

O.P. Zhirnov, E.I. Isaeva and T.E. Konakova constructed NS1-expressing plasmids, designed and performed mouse immunogenicity and H3N2 protection experiments as well as H5N3 protection experiments in chickens.

G. Thoidis contributed to the critical writing, data interpretation and manuscript preparation.

L.M. Piskareva, I.I. Akopova and A.D. Altstein manufactured NP- and M1-expressing plasmids, designed and performed H5N2 mouse protection experiments.

A. Kartashov performed statistical calculations.

P. O. Ilyinskii and A. M. Shneider were responsible for overall experimental planning, study design, data interpretation and manuscript preparation.

Conflict of interest

O.P.Z., E.I.I., T.E.K., L.M.P., I.I.A. and A.D.A. have received consulting fees from Cure Lab, Inc. G.T. and P.O.I. are employed by Cure Lab, Inc. A.M.S. is employed by Cure Lab, Inc. and owns part of its stock.

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References

1 Epstein SL, Kong WP, Misplon JA *et al.* Protection against multiple influenza A subtypes by vaccination with highly conserved nucleoprotein. Vaccine 2005;23:5404–5410.

2 Falcón AM, Marion RM, Zürcher T *et al.* Defective RNA replication and late gene expression in temperature-sensitive influenza viruses expressing deleted forms of the NS1 protein. J Virol 2004;78:3880–3888.

3 Gianfrani C, Oseroff C, Sidney J, Chesnut RW, Sette A. Human memory CTL response specific for influenza A virus is broad and multispecific. Hum Immunol 2000;61:438–452.

4 Gschoesser C, Almanzar G, Hainz U *et al.* CD4+ and CD8+ mediated cellular immune response to recombinant influenza nucleoprotein. Vaccine 2002;20:3731–3738.

5 Cox RJ, Brokstad KA, Ogra P. Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines. Scand J Immunol 2004;59:1–15.

6 Chen W, Anton LC, Bennink JR, Yewdell JW. Dissecting the multifactorial causes of immunodominance in class I-restricted T cell responses to viruses. Immunity 2000;12:83–93.

7 Okuda K, Ihata A, Watabe S *et al.* Protective immunity against influenza A virus induced by immunization with DNA plasmid containing influenza M gene. Vaccine 2001;19:3681–3691.

8 Ulmer JB. Influenza DNA vaccines. Vaccine 2002;20(Suppl. 2):S74–S76.

9 Epstein SL, Stack A, Misplon JA *et al.* Vaccination with DNA encoding internal proteins of influenza virus does not require CD8(+) cytotoxic T lymphocytes: either CD4(+) or CD8(+) T cells can promote survival and recovery after challenge. Int Immunol 2000;12:91–101.

10 Epstein SL, Tumpey TM, Misplon JA *et al.* DNA vaccine expressing conserved influenza virus proteins protective against H5 N1 challenge infection in mice. Emerg Infect Dis 2002;8:796–801.

11 Gorman OT, Bean WJ, Kawaoka Y, Webster RG. Evolution of the nucleoprotein gene of influenza A virus. J Virol 1990;64:1487–1497.

12 Ito T, Gorman OT, Kawaoka Y, Bean WJ, Webster RG. Evolutionary analysis of the influenza A virus M gene with comparison of the M1 and M2 proteins. J Virol 1991;65:5491–5498.

13 De Filette M, Min Jou W, Birkett A *et al.* Universal influenza A vaccine: optimization of M2-based constructs. Virology 2005;337:149–161.

14 De Filette M, Ramme A, Birkett A *et al.* The universal influenza vaccine M2e-HBc administered intranasally in combination with the adjuvant CTA1-DD provides complete protection. Vaccine 2006;24:544–551.

15 Fiers W, De Filette M, Birkett A, Neirynck S, Min Jou W. A ''universal'' human influenza A vaccine. Virus Res 2004;103:173–176.

16 Ozaki T, Yauchi M, Xin KQ, Hirahara F, Okuda K. Cross-reactive protection against influenza A virus by a topically applied DNA vaccine encoding M gene with adjuvant. Viral Immunol 2005;18:373–380.

17 Chen Z, Kadowaki S, Hagiwara Y *et al.* Protection against influenza B virus infection by immunization with DNA vaccines. Vaccine 2001;19:1446–1455.

18 Chen Z, Sahashi Y, Matsuo K *et al.* Comparison of the ability of viral protein-expressing plasmid DNAs to protect against influenza. Vaccine 1998;16:1544–1549.

19 Chen Z, Yoshikawa T, Kadowaki S *et al.* Protection and antibody responses in different strains of mouse immunized with plasmid DNAs encoding influenza virus haemagglutinin, neuraminidase and nucleoprotein. J Gen Virol 1999;80:2559–2564.

20 Donelan NR, Basler CF, García-Sastre A. A recombinant influenza A virus expressing an RNA-binding-defective NS1 protein induces high levels of beta interferon and is attenuated in mice. J Virol 2003;77:13257–13266.

21 Geiss GK, Salvatore M, Tumpey TM *et al.* Cellular transcriptional profiling in influenza A virus-infected lung epithelial cells: the role of the nonstructural NS1 protein in the evasion of the host innate defense and its potential contribution to pandemic influenza. Proc Natl Acad Sci U S A 2002;99:10736–10741.

22 Salvatore M, Basler CF, Parisien J-P *et al.* Effects of influenza A virus NS1 protein on protein expression: the NS1 protein enhances translation and is not required for shutoff of host protein synthesis. J Virol 2002;76:1206–1212.

23 Wang X, Li M, Zheng H *et al.* Influenza A virus NS1 protein prevents activation of NF-kappaB and induction of alpha/beta interferon. J Virol 2000;74:11566–11573.

24 Zhirnov OP, Konakova TE, Wolff T, Klenk HD. NS1 protein of influenza A virus down-regulates apoptosis. J Virol 2002;76:1617–1625.

25 Boon AC, de Mutsert G, Graus YM *et al.* The magnitude and specificity of influenza A virus-specific cytotoxic T-lymphocyte responses in humans is related to HLA-A and -B phenotype. J Virol 2002;76:582–590.

26 Basler CF, Reid AH, Dybing JK *et al.* Sequence of the 1918 pandemic influenza virus nonstructural gene (NS) segment and characterization of recombinant viruses bearing the 1918 NS genes. Proc Natl Acad Sci U S A 2001;98:2746–2751.

27 Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 1991;108:193–199.

28 Zhirnov OP. Isolation of matrix protein M1 from influenza viruses by acid-dependent extraction with nonionic detergent. Virology 1992;186:324–330.

29 Smirnov YA, Lipatov AS, Van Beek R, Gitelman AK, Osterhaus AD, Claas EC. Characterization of adaptation of an avian influenza A (H5 N2) virus to a mammalian host. Acta Virol 2002;44:1–8.

30 Altstein AD, Gitelman AK, Smirnov YA *et al.* Immunization with influenza A NP-expressing vaccinia virus recombinant protects mice against experimental infection with human and avian influenza viruses. Arch Virol 2006;151:921–931.

31 Donnelly JJ, Friedman A, Martinez D *et al.* Preclinical efficacy of a prototype DNA vaccine: enhanced protection against antigenic drift in influenza virus. Nat Med 1995;1:583–587.

32 Jameson J, Cruz J, Ennis FA. Human cytotoxic T-lymphocyte repertoire to influenza A viruses. J Virol 1998;72:8682–8689.

33 Gao W, Soloff AC, Lu X *et al.* Protection of mice and poultry from lethal H5N1 avian influenza virus through adenovirus-based immunization. J Virol 2006;80:1959–1964.

34 Wesley RD, Tang M, Lager KM. Protection of weaned pigs by vaccination with human adenovirus 5 recombinant viruses expressing the hemagglutinin and the nucleoprotein of H3 N2 swine influenza virus. Vaccine 2004;22:3427–3434.

