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Subcutaneous immunization with recombinant adenovirus expressing influenza A nucleoprotein protects mice against lethal viral challenge

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Current influenza vaccines mainly induce strain-specific neutralizing antibodies and need to be updated each year, resulting in significant burdens on vaccine manufacturers and regulatory agencies. Genetic immunization strategies based on the highly conserved nucleoprotein (NP) of influenza have attracted great attention as NP could induce heterosubtypic immunity. It is unclear, however, whether different forms of vectors and/or vaccination regimens could have contributed to the previously reported discrepancies in the magnitude of protection of NP-based genetic vaccinations. Here, we evaluated a plasmid DNA vector (pNP) and a recombinant adenovirus vector (rAd-NP) containing the NP gene through various combinations of immunization regimens in mice. We found that pNP afforded only partial protection even after four injections, with full protection against lethal challenge achieved only with the fourth boost using rAd-NP. Alternatively, only two doses of rAd-NP delivered subcutaneously were needed to induce an enhanced immune response and completely protect the animals, a finding which, to our knowledge, has not been reported before.

Introduction

Influenza is a highly contagious viral respiratory tract infection and a serious burden to public health. Current influenza vaccines largely induce strain-specific neutralizing antibodies against hemagglutinin (HA) and neuraminidase (NA) proteins.[1,2] When these vaccines match currently circulating strains they can provide substantial protection against influenza virus in a healthy population.[2-4] However, the high antigenic variability of both HA and NA necessitates annual vaccine formulation and evaluation.[5,6] Thus, alternative vaccination strategies which can provide broad immunity against various strains and subtypes, so called heterosubtypic immunity, are of great benefit.[7] It has been suggested that such immunity is induced by conserved viral components such as the nucleoprotein (NP) during influenza A virus infection in humans.[8]

Gene-based vaccination represents an exciting means of inducing protective immunity against viral infections. It offers many advantages compared with traditional vaccines including simplicity of production with high purity and the ability to induce both humoral and cellular immunity.[9-11] While some investigators report that genetic immunization with the highly conserved influenza NP through plasmid DNA or recombinant viral vectors induce heterosubtypic immunity,[1,12-15] other recent works suggest that NP based vaccines can only elicit limited immune responses and protection in animals.[16,17] Given that the previously published studies by various groups employed different vectors and/or immunization strategies, we investigated a combination of genetic immunization regimens in animal protection experiments and assessed the timing and nature of immune responses against influenza NP.

Results

Induction of immune response in mice. We compared the different vaccination strategies by analyzing NP-specific IgG titers in serum (**Fig. 1A**). To our surprise and in contrast to previously reported findings[18] immunization with the first two doses of pNP failed to induce any Ab responses in mice (**Fig. 1A** and days 14 and 28). It was only after a third dose of pNP that mice started to show marginally detectable levels of Abs (**Fig. 1A** and day 42). A fourth dose of pNP immunogen was able to elicit significant levels of Abs compared with control groups, but boosting with rAd-NP generated at least a two-fold increase in Abs on day 56 (**Fig. 1A** and day 56). On the other hand, mice immunized with rAd-NP showed significantly elevated levels of NP-specific Abs even after a single dose (**Fig. 1A** and days 14 and 28). Immunization with rAd-NP induced significant Ab

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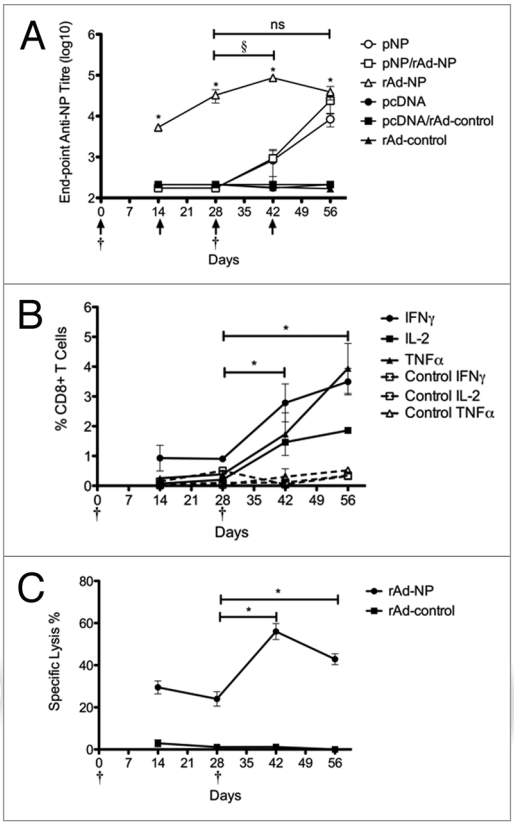

Figure 1. Recombinant adenovirus immunization induces strong immune response in mice. (A) Anti-NP Abs. For DNA immunization, mice were injected with 4 doses of either pNP or pcDNA on days 0, 14, 28 and 42. For pNP prime/rAd-NP boost, mice which received 3 doses of pNP or pcDNA were boosted with either rAd-NP or rAd-control on day 42, respectively. For adenovirus only regimen, mice were immunized with two doses on days 0 and 28 of either rAd-NP or rAd-control. Sera from immunized and control mice were collected every two weeks and subjected to ELISA to determine end-point anti-NP titers. Immunization with rAd-NP alone elicited significant antibody responses compared with controls and pNP even after a single dose, but there were no significant differences on day 56 between rAd-NP and pNP prime/rAd-NP boost immunization. Data are shown as the mean of the end-point titers of the total IgG ± SEM * and §indicate a p-value of < 0.05, and ns indicates a p-value of > 0.05 (non-significant). ↑indicates the immunization schedule for pNP and pNP-NP/rAd-NP, while †indicates the immunization schedule for rAd-NP. (B) $CD8^+$ IFNγ, TNFα, IL-2 responses to rAd as measured by intracellular cytokine staining. Intracellular cytokines were measured in $CD8^+$ T cells stimulated with synthetic NP peptide TYQ RTR ALV (restricted to H-2Kd). Responses were measured from splenocytes collected from mice immunized with rAd-NP or rAd-control every two weeks. Boosting with rAd-NP significantly enhanced cytokines in CD8+ T cells on day 42 and 56. Results for IFNγ, TNFα and IL-2 were calculated as percent of $CD8^+$ T cells. Results are shown as mean ± SEM *indicates a p-value of < 0.05 and † indicates the immunization schedule. (C) Immunization with rAd-NP induces strong CTL response. Splenocytes from mice immunized with rAd-NP or rAd-control were collected every two weeks and restimulated in vitro for 5 d with synthetic NP peptide TYQ RTR ALV. The CTL responses were measured in a LDH cytotoxicity assay against P815 cells pulsed with the same peptide. Boosting with rAd-NP significantly enhanced CTL response on day 42 and 56. The results are means of two experiments ± SEM *indicates a p-value of < 0.05 and †indicates the immunization schedule.

response at all time points compared with all groups except for mice from pNP prime/rAd-NP boost group (**Fig. 1A** and day 56). Moreover, immunization with two doses of rAd-NP induced strong T-cell responses as shown by the significant increase in antigen-specific IFNγ, TNFα, IL-2 (**Fig. 1B**) as well as in the antigen-specific CTL response as measured by the percentage of specific lysis (**Fig. 1C**) in a similar manner to previous reports on DNA and DNA prime/rAd boost regimens.[1,19] These results demonstrate that immunization with rAd-NP alone is as effective as pNP prime/rAd-NP boost but more efficient than pNP in inducing both humoral and cellular responses. Most importantly, two doses of rAd-NP were required to elicit strong humoral and cellular immune responses.

Protection against heterologous challenge. Next we evaluated the different immunization regimens for their ability to induce protection against influenza PR8 virus challenge. Mice immunized with either pNP prime/rAd-NP boost or two doses of rAd-NP were completely protected after viral challenge, whereas mice which received pNP or single-dose rAd-NP only showed survival rates of 50% and 40% respectively (**Fig. 2A**). Protected animals also significantly maintained their weight, in contrast to mice from pNP or single rAd-NP groups which lost up to 20% of their total body weight within 8 d after challenge (**Fig. 2B**). All animals in the control groups suffered from severe clinical symptoms and weight loss (**Fig. 2B**) and died between 5–9 d after viral challenge (**Fig. 2A**). As expected, mice immunized with either pNP prime/rAd-NP boost or two rAd-NP doses showed significantly lower virus titers in lungs on day 6 post-challenge compared with controls as well as to pNP and single rAd-NP groups (**Fig. 2C**). Specifically, they showed a 40-fold reduction in viral titers compared with control groups and a 10-fold reduction compared with pNP and single rAd-NP groups. On the other hand, immunization with pNP or one rAd-NP dose only reduced viral titers by 4-fold (**Fig. 2C**). Although pNP prime/rAd-NP boost strategy is as effective as two rAd-NP doses (**Fig. 2**), the former strategy requires 3 pNP injections, followed by another boost of rAd-NP. In summary, these results indicate that immunization with two doses of rAd-NP alone is the simpler procedure, and provides complete heterosubtypic protection against influenza PR8 virus similar to pNP prime/rAd-NP boost in mice.

Discussion

Influenza vaccines inducing heterosubtypic immune responses have received increasing attention because of the potential to eventually replace the annual vaccines. While identification and characterization of broadly reactive or neutralizing antibodies against conserved regions in influenza HA[20-26] and NA[27,28] may represent an important step toward such a goal, most of these Abs either have limited neutralization potency or are restricted to certain subtypes. Most importantly, current vaccination and natural influenza infection both mainly result in the recognition of the highly variable-immunodominant head domains in HA and NA which shield the more conserved regions in these two proteins.[29,30] Thus, vaccination against HA and

NA may not lead to the development of universal protection. On the other hand, internal proteins such as NP are highly conserved among all influenza subtypes. Several studies have shown that immunization of animals with NP induces strong immune responses and protects against various influenza A subtypes,[1,12-15,31] while other, more recent publications, showed some contradictory results.[16,17] To examine the role of the vectors and/or immunization regimens in the effectiveness of NP based vaccines, we investigated the protection induced by various combinations of these vectors and vaccination regimens. We are not surprised to find that pNP immunization alone was the least effective, with four injections needed to induce significant anti-NP Ab responses and only affording partial protection (**Figs. 1 and 2**). Noticeably, three doses of NP DNA vaccine were reported to provide full protection by other investigators,[19] a vaccination approach in which the DNA vector might be more efficient than the commercial DNA plasmid (pcDNA-3) we employed here although the efficient expression of NP by the commercial DNA vector has been detected (**Sup. Material**). Still, if NP DNA vaccination alone were to be considered, a single prime followed by the same DNA vector does not appear to be enough based on the results presented here and elsewhere.[19] As far as adenoviral vector for NP delivery is concerned, although we found that one injection of rAd-NP could elicit a strong Ab response, two injections of rAd-NP are still required for complete protection against viral challenge, a finding which, to the best of our knowledge, has not been reported in the literature. We did not address the issue relating to the type of immune responses elicited by DNA vaccination as it was already well documented that DNA immunization may preferentially induce cellular immunity.[19] In our work, it is however clear that the second injection of rAd-NP substantially enhanced both humoral and cellular immune responses, which were shown to correlate with complete protection of the animal from lethal challenge (**Figs. 1 and 2**). While the rAd-NP induced humoral immunity may still play an important role in protection,[32] our data presented here also highlight the importance of cell-mediated immune responses in NP-based vaccination strategy.

Recombinant Ad vectors are still considered among the safest and most effective vaccine vectors due to their potent immunogenicity and ability to induce strong immune responses. However, innate and pre-existing immunity against rAd5 may lead to acute toxicity and limited efficacy of rAd-based vaccines[33] especially after two doses of rAd5 vector. Thus, development of strategies to reduce Ad antigenicity and to overcome pre-existing immunity is of great importance. Several approaches have been considered including usage of modified Ad5,[34] chimeric Ads[35] or non-human origin Ad serotypes.[36,37]

Nevertheless, despite these interesting findings, the ideal vaccine approach would only require a single dose of rAd containing highly conserved genes such as NP and matrix (M2e) through intranasal delivery.[38] However, live adenoviruses have been reported to inadvertently invade the brain via the olfactory bulb upon intranasal administration.[39,40] Thus, it may be useful to consider adjuvants which preferentially boost cellular immune responses so that one single injection might afford effective protection.

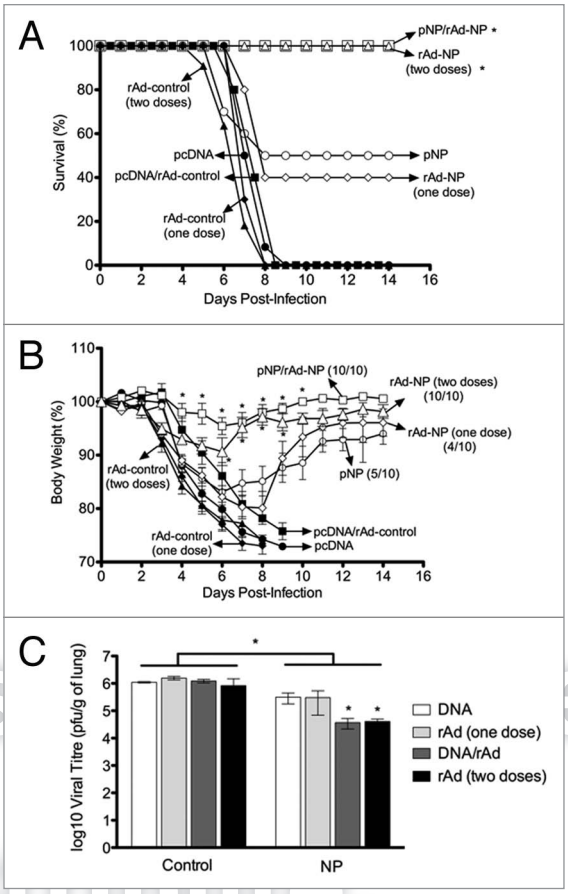

Figure 2. Two doses of rAd-NP and pNP prime/rAd-NP boosting provide superior protection after viral challenge in mice. (A) Survival of immunized mice after PR8 virus challenge. Data are presented as a percentage of surviving animals at each time point compared with the initial number of animals in each group. Mice boosted with rAd-NP after pNP priming or immunized with two doses of rAd-NP showed significant survival ($p < 0.0001$) compared with mice immunized with either four doses of pNP or a single dose of rAd-NP as well as control groups. (B) Body weights of the challenged mice. Weight loss is expressed as percentage of animal weight at each time point from their initial body weight. Animals immunized twice with rAd-NP or with pNP prime/rAd-NP boost significantly maintained their body weight compared with other immunized groups which demonstrated significant loss in weight. *indicates a p-value of < 0.05. (C) Lung viral titers. Immunization with both pNP prime/rAd-NP boost and two doses of rAd-NP strategies significantly reduced viral titers in lungs compared with other groups. There is no statistical difference between groups received wither pNP prime/rAd-NP boost or two doses of rAd-NP. *indicates a p-value of < 0.05.

Materials and Methods

Cell lines, enzymes and influenza A virus. Mouse fibroblast NIH-3T3 (ATCC: CRL-1658) and Madin-Darby Canine Kidney (MDCK) cells were obtained from the American Type

Culture Collection and human embryonic kidney QBI-HEK 293A cells were purchased from Qbiogene Inc., (Carlsbad, CA). Cells were grown and cultured in Dulbecco's Modified Eagle's Medium (DMEM) as previously described in reference 41. All restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Pickering, ON). Influenza A/Puerto Rico/8/34(H1N1) was used in this study for viral challenge (hereafter referred to as PR8). Virus stock was propagated at 37°C in the allantoic cavities of 10-d-old embryonated hen eggs for 24 h. Allantoic fluid was clarified by centrifugation, aliquoted and stored at -80°C until used. Virus was titrated in MDCK cells as previously described in reference 41.

DNA plasmids. Plasmid expressing influenza NP (pNP) was generated by cloning the NP gene from Influenza A/duck/Yokohama/aq10/2003(H5N1) (GeneBank accession number AB212281) into the *NotI* and HindIII sites of a pcDNA3.1 mammalian expression vector. Bulk endotoxin-free preparations of pNP as well as the empty control plasmid (pcDNA) were prepared using a plasmid Giga purification kit (Qiagen, Toronto, ON). Endotoxin level was confirmed to be < 1 EU.

Recombinant adenoviruses. Recombinant adenovirus type 5 expressing influenza NP (rAd-NP) or empty control vector (rAd-control) were generated using AdenoVator™ Adenoviral Expression System with pAdenoVator-CMV5(Cuo)-IRES-GFP transfer vector according to the manufacturer's instructions (Qbiogene Inc.). Briefly, *PmeI*-linearized transfer vector containing the NP gene from Influenza A/duck/Yokohama/aq10/2003(H5N1) or empty transfer vector were co-transformed with a ΔE1/E3 Ad5-backbone containing plasmid into *E. coli* cells (BJ5183) for homologous recombination. The resulting recombinant plasmids were transfected into the QBI-HEK 293A packaging cell line for generation of recombinant adenoviruses (rAds). Following plaque purification, rAds were amplified to high titers in QBI-HEK 293A cells, purified, titrated and stored at -80°C until used.

NP expression in cell culture. NIH-3T3 cells were transiently transfected with pNP or pcDNA using Fugene 6 (Roche, Laval, QC) as previously described in reference 37, or transduced with rAd-NP or rAd-control. Forty-eight hours later, cell lysates were collected and subjected to western blot analysis for protein expression using rabbit anti-NP Abs as previously described in reference 42, Both immunogens showed protein bands of expected NP size, 57 kDa, (see **Sup. Fig.**).

Mice. Six to eight-week-old female BALB/c mice were purchased from Charles River Laboratories International (Wilmington, MA). The animals were maintained in the animal facility of Health Canada (Food Directorate, Scientific Services Division, Ottawa, ON). All animal experiments were conducted in accordance with Health Canada institutional guidelines and with the approval of the Animal Care and Use Committee.

Immunization and challenge studies. Mice were subcutaneously immunized with pNP alone, pNP prime/rAd-NP boost or two doses of rAd-NP. For pNP immunization, mice were injected with four doses of 100 μg of either pNP or pcDNA dissolved in 100 μl PBS. Doses were given on days 0, 14, 28 and 42. For pNP prime/rAd-NP boost, mice which received three doses of pNP or pcDNA were boosted with 1×10^9 pfu of either rAd-NP or rAd-control on day 42, respectively. For adenovirus only regimen, mice were immunized with two doses of 1×10^9 pfu of either rAd-NP or rAd-control on days 0 and 28. Two weeks after pNP and pNP prime/rAd-NP boost, or 4 weeks after rAd-NP immunization, all mice were lightly anesthetized with CO_2 and inoculated intra-nasally with 25 μl (10 LD_{50}) of PR8 virus diluted in PBS. In some experiments, mice were challenged 4 weeks after a single dose of rAd-NP immunization. All challenge experiments used 10 mice per group; 7 mice were monitored for body weight loss and survival for 14 d, and 3 mice were euthanized on day 6 post-challenge for lung viral titration. Each experiment was repeated at least twice.

ELISA for anti-NP Abs measurement. Serum samples were collected from immunized mice on days 0, 14, 28, 42 and 56 to determine antibody response. Total anti-NP IgG antibodies was determined by ELISA. Briefly, 96-well plates (Nunc, Mississauga, ON) were coated overnight, at 4°C, with 100 μl of 4 μg/ml of purified recombinant NP protein in PBS per well. Plates were then washed 6 times with PBS containing 0.05% Tween-20 (PBS-T), followed by blocking with 5% skim milk in PBS-T for 1 h at 37°C. After washing 6 times with PBS-T buffer, serum samples were added in a 2-fold serial dilution starting from 1:100 and incubated at 37°C for 1 h. After extensive washing, peroxidase-conjugated rabbit anti-mouse IgG Abs were added at concentrations recommended by the supplier (GE Healthcare, Baie d'Urfe, QC) for 1 h at 37°C. After 6 washes with PBS-T, tetramethylbenzidine (TMB) substrate (Cell Signaling Technology, Inc., Danvers, MA) was added for 30 min for colorimetric development and the reaction was stopped with 0.16 M sulfuric acid. Absorbance was read spectrophotometrically at 450 nm. End-point titers were determined with a cut-off defined as the mean of pre-bleed samples plus three SD.

Intracellular cytokine staining (ICS). One million splenocytes from mice immunized with rAd-NP or rAd-control were re-stimulated with 5 μg/ml of synthetic NP peptide TYQRTRALV (restricted to H-2Kd) in the presence of 1 μg/ml Golgiplug (eBiosciences, San Diego, CA) for 6 h. Cells were then stained with CD8α-APC-eFluor780, IFNγ-PerCPcy5.5, TNFα-PE and IL2-PeCy7 antibodies (eBiosciences). A BD LSRII flow cytometer was used for data acquisition and analysis was completed with Flow Jo, Version 8.8.4 (Tree Star Inc., Ashland, OR). Unstained cells and single stained compensation beads (BD Biosciences, Mississauga, ON) were used as controls for background fluorescence and false positives due to fluorochrome bleeding. Results for IFNγ, TNFα and IL-2 were calculated as percent of $CD8^+$ T cells.

CTL assay. Approximately, 3×10^7 splenocytes from mice immunized with rAd-NP or rAd-control were cultured to generate effector CTL as previously described in reference 41. After 5 d of culture, cytotoxic activity was measured by LDH release assay using NP peptide-pulsed P815 targets (H-2d) and the percentage of specific lysis was calculated.

Lung viral titration. Lungs were harvested from challenged mice 6 d post-challenge, homogenized and used for viral titration

by plaque assay in MDCK cells as described in reference 40. Titers were expressed as $\log_{10}$ pfu/g of tissue.

Statistics. One-way Analysis of Variance (ANOVA) with Bonferroni post-test was used to compare data from the different groups. Statistical significance in survival was tested using the log-rank test. All statistical analysis was conducted using GraphPad Prism software (San Diego, CA). p-values < 0.05 were considered statistically significant. Results are presented as mean ± SEM.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Note

Supplemental material can be found at:
www.landesbioscience.com/journals/hv/article/19109

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