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Cellular immune response in the presence of protective antibody levels correlates with protection against 1918 influenza in ferrets

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ABSTRACT

The identification of immune correlates of protection against highly pathogenic human-adapted influenza is instrumental in the development of the next generation of vaccines. Towards this, ferrets received either one dose of a conventionally produced vaccine, two inoculations of a hemagglutinin (HA)-expressing DNA vaccine, or a prime-boost regimen of the DNA vaccine followed by injection of a HA-expressing adenoviral vector. In addition to the antibody response, ferret-specific interferon-gamma (IFN-γ) ELISpot and flow cytometry assays were developed to follow the cellular immune response. Animals that received the conventional vaccine mounted a humoral response, while the DNA vaccinated groups also developed IFN-γ producing T cells. Upon challenge with the matched highly pathogenic A/South Carolina/1/18 H1N1 influenza A virus, the conventionally vaccinated group developed moderate to severe signs of disease, whereas the DNA vaccinated animals experienced mild disease. In the presence of an antibody response within the protective range, the extent of the T cell response correlated more accurately with reduced morbidity in vaccinated ferrets.

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1. Introduction

Despite annual vaccination programs, up to 15% of the population is affected by seasonal influenza, and more than 300,000 deaths worldwide are attributed to this disease and its ensuing complications [1]. In a pandemic situation, the lack of pre-existing immunity in the population against a newly emerging strain can lead to a dramatic increase in infection rates and substantial economic losses [2–4]. Moreover, there is a general consensus that, due to the high infectivity and short incubation period, a widely available efficient vaccine will be the most efficient clinical modality to control future pandemics caused by more virulent influenza viruses [5].

The traditional influenza vaccine contains inactivated virus grown in embryonated chicken eggs. In addition to conventional influenza vaccines, several experimental immunization strategies based on virus-like particles, recombinant adenoviruses, or DNA, are being developed with the hope of inducing a broader immune response, which may improve protection against highly pathogenic influenza viruses [6,7]. These vaccine strategies are initially evaluated in mice and ferrets, two widely used animal models of influenza infection. A main parameter monitored during these studies is the B-cell response, which is well documented to correlate with protection. The increased influenza mortality seen in mice that lack B cells, and the positive correlation between vaccination-induced antibody titers and protection from clinical disease constitute the basis for the use of quantitative post-vaccination antibody levels as the endpoint for clinical protection [8,9]. Detectable serum antibody titers equal to or above 1/40, as determined by hemagglutination inhibition (HI) or neutralizing antibody (NAB) assays, are considered predictive of a favorable clinical outcome following an exposure to influenza [10,11]. Of note, the contribution of the cellular immune response to the establishment of protective immunity against influenza virus infections is increasingly recognized [12–14]. Interestingly, the avian influenza hemagglutinin (HA) was previously

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shown to stimulate a higher T-cell response than the neuraminidase (NA), nucleoprotein (NP) or the matrix protein M2 in immunized mice as well as in infected nonhuman primates [15].

Ferrets are considered an excellent model for influenza vaccine efficacy assessments since they are naturally susceptible to human influenza viruses and develop a disease comparable to that observed in humans [16–18]. As outbred animals, their immune response diversity somewhat mimics the immune variability encountered in the human population [19]. However, this diversity makes the evaluation of the cellular immune response more challenging. The aim of the present study was to better understand the contribution of both arms of the immune response to protection and infection control. Towards this, mice and ferrets were immunized with vaccine strategies expected to generate different levels of protection and the resulting T and B cell responses were evaluated. The different immune responses were then correlated with respective clinical outcomes following challenge with A/South Carolina/1/18 (H1N1), a highly pathogenic human-adapted influenza virus [20,21].

2. Materials and methods

2.1. Virus and vaccine production

The influenza A virus H1N1 A/South Carolina/1/18 (H1N1-1918) was amplified in Madin-Darby canine kidney (MDCK) in Dulbecco's modified Earle's medium (DMEM, Invitrogen) with 2 μg/ml tolylsulfonyl phenylalanyl chloromethyl keton (TPCK)-trypsin (Sigma). The titers were determined by plaque assay and expressed as PFU per ml.

For the generation of the DNA vaccine, the H1N1-1918 (HA18) cDNA was first codon-optimized, and the gene was reconstructed from overlapping 40mer oligonucleotide primers. The resulting HA18 gene was then inserted into a pCAG vector, containing a chicken-ß-actin promoter resulting in the DNA vaccine construct pCAGα-HA18 (DNA-HA18). All DNA-based vaccine preparations were produced and purified with an endotoxin-free gigaprep kit (Qiagen). The expression cassette, including promoter, was also excised and inserted into pAdenoX (AdHu5, Clontech, BD Biosciences) via transfer plasmid pShuttle2 (Clontech). The resulting Ad-HA18 construct was transfected into human embryonic kidney (HEK) 293 cells (Clontech) and recovered after twenty days. Recombinant virus stocks were produced in HEK 293 cells, and the virus was purified using a cesium chloride density gradient. The Ad-HA18 vector preparations were titrated using both the AdenoX rapid titre kit (BD Biosciences) and optical density measurement at 260 nm.

For the conventional vaccine, a recombinant virus containing the HA18 and NA18 genes in the genetic background of the vaccine production strain A/Puerto Rico/8/34 (PR8) was generated using the eight-plasmid system [22]. This HA/NA18:PR8 reassortant was amplified in MDCK cells in DMEM with 2 μg/ml TPCK-trypsin. After clarification of the supernatant by low-speed centrifugation, the virus was pelleted on a 30% sucrose cushion in a SW32Ti rotor at 25,000 rpm. The pellet was resuspended in calcium borate buffer (145 mM NaCl, 10 mM $CaCl_2$, 2.5 mM sodium borate, 20 mM boric acid; pH 7.0) and subsequently inactivated with 0.1% formaldehyde in phosphate-buffered saline (PBS, Invitrogen) for 3 days. To quantify the HA content, an aliquot of the inactivated sample and the seasonal commercial vaccine with known HA content (GSK) were separated by SDS-PAGE and HA bands were quantified after Coomassie Blue staining (Bio-Rad).

2.2. Western blot analysis

HEK 293T cells were cultured in 60 mm dishes to 80–90% confluency and transfected with DNA-HA18 by using calcium phosphate precipitation method or infected with Ad-HA18. Cells were incubated for 48–72 h and harvested for Western blot analysis as previously described [15]. The membrane was incubated with anti-sera obtained from cynomologus macaques (*Macaca fascicularis*) infected with H1N1-18 [21] and subsequently probed with an anti-monkey IgG antibody conjugated to horseradish peroxidase secondary antibody. The presence of the protein was visualized using the ECL detection kit (GE Healthcare).

2.3. Vaccination of mice and ferrets

All procedures and scoring sheets were approved by the Animal Care Committee at the National Microbiology Laboratory (NML) at the Public Health Agency of Canada (PHAC) and the Institutional Animal Care and Use Committee of the INRS – Institut Armand-Frappier, according to the guidelines set by the Canadian Council on Animal Care. All infectious work was performed in the biocontainment level 4 laboratory at NML/PHAC. Mice: Six to eight week old BALB/c mice were obtained from Charles River Canada. Groups of eight animals were immunized by intramuscular injection of 50 μg of DNA-HA18, 10^{10} PFU of Ad-HA18, or 4 μg HA equivalent of the HA/NA18:PR8 formaldehyde-inactivated vaccine. Four animals of each group were sacrificed 10 days after immunization and their spleens were harvested. Serum samples were collected after 4 weeks prior to challenge from all remaining animals. Ferrets: Sixteen week-old male ferrets (Marshall Farms) without antibodies against circulating influenza viruses were used for this study. Groups of six animals were immunized intramuscularly with either two electroporations of 500 μg DNA-HA18 into the neck muscles 4 weeks apart (DNA prime-boost), one injection of 500 μg of DNA-HA18 into the neck muscles followed 4 weeks later by one injection of 10^{10} PFU of Ad-HA18 (DNA prime-viral boost), or one injection of a formalin-inactivated vaccine containing 4 μg of HA18 at the time of the second inoculations. Previous studies reported the lack of priming effect of adenovirus or DNA vaccination coding for irrelevant antigens on the response to influenza antigens [23–25], thus two injections of 500 μl PBS injections were used for the control group. Heparinized blood was collected weekly during the immunization period, and serum samples were collected three weeks after each injection and four weeks after the challenge.

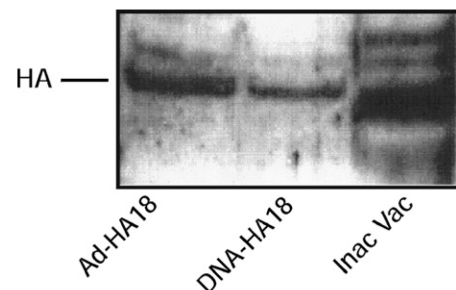

Fig. 1. Hemagglutinin (HA) expression associated with the different vaccines. Equivalent protein concentrations of lysates of HEK293 cells infected with Ad-HA18 or transfected with DNA-HA18, or the inactivated vaccine (Inac Vac) were loaded on polyacrymalide gel for Western blot analysis. HA was detected using an antiserum obtained from cynomologus macaques infected with H1N1-1918.

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