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Towards a universal vaccine for avian influenza: Protective efficacy of modified Vaccinia virus Ankara and Adenovirus vaccines expressing conserved influenza antigens in chickens challenged with low pathogenic avian influenza virus

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ABSTRACT

Current vaccines targeting surface proteins can drive antigenic variation resulting either in the emergence of more highly pathogenic viruses or of antigenically distinct viruses that escape control by vaccination and thereby persist in the host population. Influenza vaccines typically target the highly mutable surface proteins and do not provide protection against heterologous challenge. Vaccines which induce immune responses against conserved influenza epitopes may confer protection against heterologous challenge. We report here the results of vaccination with recombinant modified Vaccinia virus Ankara (MVA) and Adenovirus (Ad) expressing a fusion construct of nucleoprotein and matrix protein (NP+M1). Prime and boost vaccination regimes were trialled in different ages of chicken and were found to be safe and immunogenic. Interferon-γ (IFN-γ) ELISpot was used to assess the cellular immune response post secondary vaccination. *In ovo* Ad prime followed by a 4 week post hatch MVA boost was identified as the most immunogenic regime in one outbred and two inbred lines of chicken. Following vaccination, one inbred line (C15I) was challenged with low pathogenic avian influenza (LPAI) H7N7 (A/Turkey/England/1977). Birds receiving a primary vaccination with Ad-NP+M1 and a secondary vaccination with MVA-NP+M1 exhibited reduced cloacal shedding as measured by plaque assay at 7 days post infection compared with birds vaccinated with recombinant viruses containing irrelevant antigen. This preliminary indication of efficacy demonstrates proof of concept in birds; induction of T cell responses in chickens by viral vectors containing internal influenza antigens may be a productive strategy for the development of vaccines to induce heterologous protection against influenza in poultry.

1. Introduction

As zoonotic pathogens with pandemic potential, avian influenza (AI) viruses represent a significant threat to human health. Outbreaks of AI in domestic birds also place a significant financial burden on the global poultry industry, threatening food security by raising food prices in the face of increasing world protein demand. Commercial vaccines targeting AI surface proteins such as haemagglutinin offer limited protection against heterologous viral strains. As vaccination for AI has become more common, the level of antigenic drift in avian viruses has increased. In Mexico, long term vaccination coupled with a failure to eradicate low pathogenic H5N2 AI has resulted in the observation that similar levels of antigenic drift can now be seen in avian viruses compared with human viruses [1]. Vaccination without eradication increases the likelihood that antigenically distinct and potentially more virulent strains of AI will arise and a major goal of vaccination is therefore to produce sterilising immunity. An ideal AI vaccine would offer heterologous protection and eliminate shedding of infectious virus. Current vaccination strategies induce mainly humoral immunity which is not sustainably cross protective as viruses diversify away from vaccine specificity. A more effective approach aims to include the induction of T cell responses which have been shown to confer protection against heterologous influenza virus strains in humans and mice [2,3]. Recent research has shown that in human volunteers vaccination with MVA-NP+M1 successfully boosted pre-existing cellular immune responses to seasonal influenza, resulting in significantly increased *ex vivo* T cell IFN-γ ELISpot responses to NP and M1 peptides [4] and significant reduction in duration of viral shedding in vaccinated volunteers following influenza virus challenge [5].

Abbreviations: AI, avian influenza; NP+M1, fusion construct of influenza nucleoprotein and matrix protein; SFU, spot-forming units; dpb, days post boost; dpi, days post infection; wph, weeks post hatch; SPF, specific pathogen free.

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Cross reactive cell mediated responses are thought to play a similar role in the chicken although there is a paucity of literature [6]. These responses target conserved epitopes of internal antigens which are subject to lesser degrees of antigenic drift compared to antigens of the surface proteins haemagglutinin and neuraminidase. A significant benefit of a vaccine targeting conserved internal antigens is the cell mediated destruction of viral factories and hence reduction in viral load and shedding, irrespective of the variation of surface antigens. Conserved internal proteins thought to contribute to cross protection include nucleoprotein and matrix protein. Recent studies have demonstrated the utility of nucleoprotein in inducing T cell responses in chickens, although these did not investigate protection against challenge with influenza virus [7,8]. To investigate cross protective responses in chickens we have established prime and boost vaccination regimes with recombinant modified Vaccinia virus Ankara (MVA) and Adenovirus (Ad) expressing a fusion construct of nucleoprotein and matrix protein (NP + M1). M1 is a known target for T cells [3] and as a conserved internal antigen was expected to confer protection in the same way. Additionally the inclusion of another antigen increases the number of potential protective epitopes. The objectives of this study were to establish the safety and immunogenicity of the NP + M1 construct in viral vector delivery systems in domestic poultry, to investigate optimisation of regimes and to determine vaccine efficacy against challenge with low pathogenic avian influenza (LPAI).

2. Materials and methods

2.1. Experimental animals

Specific pathogen free (SPF) inbred white leghorns (Line 6_1 and 15I) and outbred Light Sussex chickens were supplied as 17–19 days embryos by the Poultry Production Unit of The Pirbright Institute, Compton laboratory. Birds were hatched and maintained in SPF containment with *ad libitum* access to water and commercial chicken feed. For influenza challenge birds were transferred to Bioflex B50 Rigid Body Poultry isolators (Bell Isolation Systems Ltd, UK) and allowed to acclimatise prior to infection. Birds were examined daily for clinical signs of disease. Experimental procedures were carried out in accordance with Ethical Review and UK Home Office requirements.

2.2. Vaccination regimes

MVA and non-replicating human Adenovirus 5 (Ad) vectors expressing a fusion of nucleoprotein (NP) and matrix protein (M1) from influenza A/Panama/2007/99 (H3N2) and Ad and MVA-vectored constructs containing GFP as irrelevant antigen controls were supplied by the Vector Core Facility at the Jenner Institute (Oxford, UK). The NP + M1 antigen comprises the complete sequences of NP and M1 from A/Panama/2007/99 fused by a 7 amino acid linker sequence. Expression of the fusion protein in MVA is controlled by the Vaccinia P7.5 promoter inserted at the thymidine kinase locus. Recombinant MVA was generated in primary chick embryo fibroblast (CEF) cells. In Ad, which is E1 and E3 deleted, the human CMV promoter is used to drive expression of the fusion protein. Recombinant Ad was generated in 293 cells. These are replication-deficient viral vectors that do not persist *in vivo* following immunisation, and it is expected that antigen expression lasts from a few hours (MVA) to a few days (Ad5). Priming vaccination was performed *in ovo* at 17–19 days of incubation. 1×10^9 IU (infectious units) Ad-NP + M1 or Ad-GFP in 100 μL of sterile PBS was injected into the amniotic cavity with a 23 gauge needle to an approximate depth of 1 in. Boost vaccination with 1×10^7 pfu (plaque forming units) MVA-NP + M1 or MVA-GFP in 100 μL of sterile PBS was administered by intramuscular (IM) injection (see appropriate results section text for precise regime details). Quantification of Ad was performed by infecting cell lines and then staining for foci of infection. This quantifies infectious virus particles per volume. IU for adenovirus is stated rather than PFU as we are assaying the ability of the virus particles to infect a single cell rather than to form a plaque resulting from the initial infection of a single cell followed by spreading of the infection to surrounding cells.

2.3. Sampling/swabbing

Swabbing of the buccal and cloacal cavities of experimental birds was carried out every 2–3 days for 2 weeks post infection with sterile polyester tipped swabs (Fisher Scientific, UK). Swab tips were transferred to 1 ml viral transport medium containing antibiotics and antimycotics, vortexed briefly and medium was either frozen at −80 °C for subsequent extraction of RNA or used within 2 days for plaque assays [9].

2.4. ELISpot

Post mortem spleens were macerated in sterile PBS supplemented with 1% foetal calf serum (Invitrogen, UK) and passed through a 100 μm cell strainer (Fisher, UK) before under-laying Histopaque 1119 (Sigma, UK) and centrifugation at $400 \times g$ for 40 min. Cells at the interface were collected, washed twice in sterile PBS/1% FCS and resuspended in complete medium (RPMI with 2 mM Glutamax-I, supplemented with 10% foetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin (all Invitrogen, UK)). Cells were used immediately in ELISpot assays. Overlapping pools of 15–20-mer peptides covering the NP + M1 fusion construct sequence were used to measure T cell responses to vaccine antigens (Peptide Protein Research, Cambridge, UK). Pools of irrelevant peptides were used to control for background responses. IFN-γ ELISpot was carried out using the Chicken IFN-γ CytoSet™ (Life Technologies, UK) as described previously [10]. Briefly, 96-well multiscreen plates with Protein Binding Immobilon-P-Membrane (Millipore, Billerica,USA) were coated with 100 μL/well of 5 mg/ml mouse anti-chicken IFN-γ (Life Technologies, UK) in coating buffer (Sigma–Aldrich, UK) overnight at 4 °C. Plates were washed twice with blocking buffer (phosphate buffered saline supplemented with 2% FCS), then blocked with complete medium (see above) for 1 h at 37 °C. The blocking medium was discarded and 5×10^5 splenocytes per well were incubated overnight at 41 °C in 5% CO_2 in air, in the presence of non-specific mitogen (1 μg/ml phorbol 12-myristate 13-acetate (PMA, Sigma, UK) plus 1 μg/ml ionomycin (Sigma, UK)), recombinant NP protein (1:10 dilution of supernatant from S2 insect cells transfected with a plasmid expressing NP), beta-propiolactone inactivated LPAI (A/Turkey/England/1977/H7N7, 1×10^5 pfu/ml) or NP + M1 peptide pools (10 μg/ml) in complete medium.

2.5. LPAI virus and challenge infection

LPAI virus (A/Turkey/England/1977/H7N7) was cultured in embryonated eggs using standard methods described elsewhere [9]. Viral titre was estimated by plaque assay on Madin-Darby canine kidney (MDCK) cells, using standard techniques [11]. Challenge infection was carried out 14 days after boost vaccination, by intra-nasal inoculation of LPAI (A/Turkey/England/1977 H7N7) at a dose of 3.4×10^7 pfu in 100 μl of PBS per bird. The vaccine transgene and challenge virus NP sequences were 90.4% identical at the amino acid level. We do not have sequence data for the challenge