Vaccine 28 (2010) 1778–1785

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Seasonal influenza vaccine elicits heterosubtypic immunity against H5N1 that can be further boosted by H5N1 vaccination

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ARTICLE INFO

Article history:
Received 15 July 2009
Received in revised form
26 November 2009
Accepted 2 December 2009
Available online 16 December 2009

Keywords:
Seasonal influenza vaccine
Heterosubtypic immunity
H5N1 protection

ABSTRACT

Recent findings indicate that seasonal influenza vaccination or infection of healthy humans may contribute to heterosubtypic immunity against new influenza A subtypes, such as H5N1. Here, we investigated whether seasonal influenza vaccination in a mouse model could induce any immunity against the H5N1 subtype. It could be demonstrated that, largely due to the H1N1 component strain A/NewCaledonia/20/99, parenteral immunization of mice with a trivalent seasonal influenza vaccine elicited heterosubtype H5-reactive antibodies able to confer partial protection against H5N1 influenza virus infection. Furthermore, the trivalent seasonal influenza vaccine was found to be compatible with a whole virus H5N1 vaccine in a heterologous prime-boost immunization regimen, achieving superior efficacy compared to a single immunization with an equivalent low-dose of the H5N1 vaccine.

1. Introduction

Heterosubtypic immunity to human influenza A viruses is an established phenomenon whereby immunity induced by a virus of one influenza A subtype, or its antigens, protects against infection with a virus of a different influenza subtype [1]. This phenomenon has been well described for human influenza A viruses but little is known about the extent of heterosubtypic immunity against influenza A viruses from other species.

Currently, two influenza A subtypes, H1N1 and H3N2, are circulating in the human population, resulting in the induction of some degree of immunity in a large proportion of the population. However, continuous antigenic drift in the surface haemagglutinin protein of these human viruses means that there is a gradual decrease in the effectiveness of the immune response to protect against influenza virus infection [2]. A further consequence of this antigenic drift is the need for the annual review of the virus strains to be included in influenza vaccines. This, in turn, has led the suggestion that immunity to seasonal influenza would provide little or no effective protection against an antigenically shifted influenza virus, leading to pandemics [3–5]. Three influenza pandemics occurred in the last century: Spanish Flu in 1918, Asian Flu in 1957, and Hong Kong Flu in 1968, caused by H1N1, H2N2 and H3N2 influenza strains, respectively. However, since 1997, the spread of avian influenza caused by the highly pathogenic subtype H5N1 virus, and the concomitant infection of people resulting in a fatality rate of over 60% [6], have highlighted the threat of a new influenza pandemic.

Although it is commonly perceived that the human immune system is naïve to the avian influenza H5N1 virus, there is evidence in the literature that a certain level of heterosubtypic immunity exists against the H5N1 virus [7–16]. It has been demonstrated that healthy individuals can possess heterosubtypic memory T-cells that have the ability to recognize avian influenza H5N1 epitopes, predominantly derived from internal proteins [8,10,11]. Moreover, there have also been reports that healthy individuals can possess heterosubtypic antibodies, which could neutralize H5N1 virus infection [7,13,14,16] or have the ability to inhibit neuraminidase activities [12]. While most research has focused on natural infection-induced heterosubtypic immunity, more recently it has been demonstrated that heterosubtypic immunity against avian H5N1 influenza viruses can also be achieved by immunization with seasonal influenza vaccines as shown in both mice [17–20] and humans [13,16]. However, in these animal studies, inactivated seasonal influenza vaccine required adjuvantation with immunostimulating complexes (ISCOM), which are not licensed for human use, or required administration via the intranasal route to achieve heterosubtypic immunity. In the studies described here, the immunogenicity and efficacy of an inactivated seasonal influenza vaccine given via a more typical parenteral route was investigated to assess the potential and limitations of current vaccination strategies in providing heterosubtypic protection against a possible influenza pandemic caused by the avian H5N1 influenza

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doi:10.1016/j.vaccine.2009.12.008

virus. Furthermore, the effect of priming the immune system with an inactivated whole virus seasonal influenza vaccine on the efficacy of an inactivated whole virus H5N1 vaccine was also examined.

2. Materials and methods

2.1. Vaccine production

Viruses were provided by the Centers for Disease Control and Prevention (CDC, Atlanta, USA) or the National Institute for Biological Standards and Control (NIBSC, UK). Growth of influenza virus strains A/Vietnam/1203/2004 (H5N1; CDC#2004706280), A/NewCaledonia/20/99 (H1N1; NIBSC), A/NewYork/55/2004 (H3N2; NIBSC) and B/Jiangsu/10/2003 (B-type; NIBSC) in Vero cells in fermenters containing microcarrier cultures were described previously [21,22]. Supernatant virus was harvested, double-inactivated with formalin and by UV-irradiation and purified by sucrose gradient ultracentrifugation followed by ultra-/diafiltration. Influenza haemagglutinin (HA) antigen content was determined by single radial immunodiffusion assays [23]. All experiments using highly pathogenic infectious wild-type avian H5N1 viruses, including work with animals, were conducted according to enhanced biosafety level 3 containment procedures.

2.2. Immunizations and challenge of animals

Female outbred CD1 mice (6–8 weeks old) were used in all experiments. Mice were immunized subcutaneously (sc) with 3.75 μg of the appropriate vaccine strain or with buffer alone for control animals. Whole virus seasonal influenza vaccines were used either individually or combined as trivalent vaccines. An equivalent homologous booster dose was administered three weeks after primary immunization. Three weeks after the booster immunization, serum was collected for determining the H5-reactive IgG titre by ELISA and spleens were removed from a subset of mice for determining the frequency of interferon-γ (IFN-γ) or interleukin-4 (IL-4) secreting cells in the ELIspot assay.

To determine the effect of priming with trivalent seasonal influenza vaccine, and boosting with the whole virus H5N1 vaccine, CD1 mice were immunized (sc) with either the trivalent seasonal influenza vaccine (3.75 μg HA/strain) or with buffer. Three weeks after the primary immunization, mice were immunized (sc) with either the A/Vietnam/1203/2004 H5N1 whole virus vaccine (serial fivefold dilutions beginning at 3.75 μg HA antigen per dose) or with buffer. Three weeks after the booster immunization, serum was collected for determining the H5-reactive IgG titre by ELISA and the mice challenged intranasally with 20 μl containing a lethal dose (1×10^4 $TCID_{50}$ units = approximately 100 times the lethal dose 50% (LD_{50})) of A/Vietnam/1203/2004 H5N1 wild-type virus. Challenged mice were monitored for 14 days for disease symptoms and death.

For passive immunization experiments, test sera were produced in female outbred CD1 mice immunized 3 times (with three-week intervals) sc with the trivalent seasonal influenza vaccine (3.75 μg HA antigen of each strain) and the serum collected three weeks after the last immunization. Positive control, anti-H5N1 serum was pooled from mice immunized twice with 3.75 μg HA of the A/Vietnam/1203/2004 H5N1 vaccine, and negative control serum was pooled from naïve mice. Passive transfer was performed on three consecutive days with 400 μl of pooled serum per day administered intravenously to naïve recipient CD1 mice. One day after the last passive immunization, recipient mice were bled for serological analysis and challenged, as described before, with 1×10^4 $TCID_{50}$ of A/Vietnam/1203/2004 H5N1 wild-type virus.

Statistical analysis was performed using SAS V 8.2 for Linux to calculate the *P*-values, stratified for the experiment. Multiplicity adjustment was applied using the Bonferroni-Holm procedure. The protective dose 50% (PD_{50}) was calculated by Logit analysis using an in-house software program.

2.3. Ex vivo IFN-γ and IL-4 ELIspot assay

The frequency of IFN-γ or IL-4 secreting splenocytes was analysed using a mouse IFN-γ and IL-4 ELIspot assay following the instructions of the manufacturer (Mabtech AB, Nacka, Sweden). Serial dilutions of splenocytes from each mouse (ranging from 6×10^4 to 2×10^5 cells/well) were distributed in the wells of polyvinylidene difluoride treated 96-well plates previously coated with an anti-mouse IFN-γ or an anti-mouse IL-4 monoclonal antibody. Splenocytes were then either left unstimulated or were stimulated with an inactivated trivalent seasonal influenza or an inactivated whole virus A/Vietnam/1203/2004 (H5N1) antigen preparation at a concentration of 0.3 μg HA/ml per strain. The plates were placed in a CO_2 incubator at 37 °C. The following day, the splenocytes were discarded and after extensive washing with PBS, IFN-γ and IL-4 spots were detected by biotinylated anti-mouse IFN-γ or IL-4-specific antibody followed by addition of streptavidin alkaline phosphatase and development with BCIP/NBT substrate solution. The spots were counted using an automated ELIspot reader. The results were expressed as the number of spot-forming cells (SFC)/10^6 spleen cells. Statistical analyses were performed using a stratified bootstrap sampling method [24] to estimate the means and 95% CI for means. The *P*-values were calculated using a permutation test stratified for the experiment. All analyses were performed using R v2.7.2 for Windows.

2.4. ELISA

Haemagglutinin (HA) H5-specific serum IgG antibodies were detected by ELISA. Microtitre plates were coated with 50 ng of purified recombinant A/Vietnam/1203/2004 haemagglutinin protein (Protein Sciences, Meriden, CT, USA). Subsequent to washing and blocking, diluted serum samples were applied and reactions were detected with the horseradish peroxidase-labelled secondary IgG antibody, the ELISA was developed using O-phenylenediamine/H_2O_2. Finally, colour development was stopped by the addition of H_2SO_4, and absorbances were measured at 490 nm, using 620 nm as reference wavelength. The H5-reactive IgG titre, expressed as a reciprocal dilution, was measured as an end-point titre. All absorbance readings equal or greater than the cut-off value (4 times the mean absorbance of the negative control serum) were considered positive. For statistical analysis, end-point titres were log-transformed and geometric means calculated. The analysis was performed using SAS V 8.2 for Linux. Confidence intervals (CIs) and least square means were calculated using the MIXED procedure. Adjustment for multiplicity was applied using the Holm procedure [25] for the *P*-values, therefore the CIs are not multiplicity adjusted.

2.5. Microneutralization assay

Sera were serially diluted in twofold steps, mixed with the respective A/Vietnam/1203/2004 virus strain at a concentration of 100 $TCID_{50}$ per well, and transferred to a monolayer of Vero cells [21]. Five to seven days post-incubation, Vero cells were inspected for cytopathic effects. The neutralizing antibody titre was defined as the reciprocal serum dilution at which virus growth was 50% inhibited and was calculated by the method of Reed and Muench [26].