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The effect of gamma-irradiation conditions on the immunogenicity of whole-inactivated Influenza A virus vaccine

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ABSTRACT

Gamma-irradiation, particularly an irradiation dose of 50 kGy, has been utilised widely to sterilise highly pathogenic agents such as Ebola, Marburg Virus, and Avian Influenza H5N1. We have reported previously that intranasal vaccination with a gamma-irradiated Influenza A virus vaccine (γ-Flu) results in cross-protective immunity. Considering the possible inclusion of highly pathogenic Influenza strains in future clinical development of γ-Flu, an irradiation dose of 50 kGy may be used to enhance vaccine safety beyond the internationally accepted Sterility Assurance Level (SAL). Thus, we investigated the effect of irradiation conditions, including high irradiation doses, on the immunogenicity of γ-Flu. Our data confirm that irradiation at low temperatures (using dry-ice) is associated with reduced damage to viral structure compared with irradiation at room temperature. In addition, a single intranasal vaccination with γ-Flu irradiated on dry-ice with either 25 or 50 kGy induced seroconversion and provided complete protection against lethal Influenza A challenge. Considering that low temperature is expected to reduce the protein damage associated with exposure to high irradiation doses, we titrated the vaccine dose to verify the efficacy of 50 kGy γ-Flu. Our data demonstrate that exposure to 50 kGy on dry-ice is associated with limited effect on vaccine immunogenicity, apparent only when using very low vaccine doses. Overall, our data highlight the immunogenicity of influenza virus irradiated at 50 kGy for induction of high titre antibody and cytotoxic T-cell responses. This suggests these conditions are suitable for development of γ-Flu vaccines based on highly pathogenic Influenza A viruses.

1. Introduction

Emergence of Highly Pathogenic Avian Influenza (HPAI) strains, H5N1, H5N6, H7N9, and H9N2, represent major health concerns due to the risk of worldwide pandemics [1]. Since 2003, the World Health Organisation (WHO) reported over 800 cases of human infection with avian H5N1, with an average mortality rate of 53% [2]. Most infections with H5N1 occur via infected poultry, though rare clusters of human-human transmission have been reported between family groups in Thailand [3,4], Indonesia [5], Turkey [6], and Vietnam [4]. HPAI may gain mutations to facilitate aerosol transmission between humans, as notably, a mere 5 mutations in a laboratory H5N1 strain allowed efficient aerosol transmission between ferrets [7–9]. Existing inactivated Influenza vaccines induce strain-specific antibody responses, hence protective efficacy against emerging seasonal and pandemic strains is limited [12,13]. We reported the possible use of gamma-irradiated Influenza A virus (γ-Flu) as a vaccine candidate capable of inducing cross-protection against seasonal and pandemic virus strains [10,11,16].

To ensure sterility of irradiated influenza materials, the concept of Sterility Assurance Level (SAL) has been adopted and a value of 10^{-3} or 10^{-6} (one in a thousand or million chance of having live micro-organisms after treatment) has been arbitrarily determined and widely accepted [19]. The Australian Department of Agriculture recently considered an irradiation dose of 50 kGy as mandatory for sterilisation of highly pathogenic agents [14,15]. Considering the risk of avian Influenza pandemics, inclusion of HPAI strains may be warranted in future γ-Flu preparations; hence vaccine irradiation dose may be increased to meet the safety requirement. However, increasing irradiation dose may affect vac-

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cine efficacy. In addition, while damaging effect of γ-irradiation is dose-dependent [17,18]; the extent of structural damage is influenced by irradiation temperature [20,21,36–38]. Importantly, protein antigenicity is better maintained when virus samples are irradiated on dry-ice (DI) [23]. In the current study, we investigated the effect of irradiation dose and temperature on the immunogenicity of γ-Flu.

2. Materials and methods

2.1. Ethics statement

This study was conducted in strict accordance with Australian Code of Practice for Care and Use of Animals for Scientific Purposes (7th edition [2004], 8th edition [2013]) and South Australian Animal Welfare Act 1985. Experimental protocol approved by Animal Ethics Committee at The University of Adelaide (S-2013/014 & S-2016/036).

2.2. Cells & viruses

Influenza A virus [A/Puerto Rico/8/34 (H1N1) (A/PR8)] was grown in allantoic cavity of 10-day-old embryonated chicken eggs. Eggs injected with 10^3 $TCID_{50}$ A/PR8, incubated for 48 h at 37 °C, and chilled at 4 °C overnight. Allantoic fluid harvested, pooled and stored at −80 °C. Virus stock titrated in Madin-Darby Canine Kidney (MDCK) cells using $TCID_{50}$ assay [24] and estimated as 1.5×10^6 $TCID_{50}$/mL. Virus stock concentrated using chick erythrocytes (cRBCs) as previously described [25]. Concentrated A/PR8 stock titre estimated as 2×10^8 $TCID_{50}$/mL. For Haemagglutination Assay, live or irradiated stocks were serially diluted in PBS using 96-well round-bottom plate and 0.8% cRBCs in PBS added. Plates were incubated at 4 °C and haemagglutination patterns analysed 24 h later.

2.3. Vaccine preparations

A/PR8 stocks inactivated by exposure to γ-radiation from ^{60}Co irradiation facility at Australian Nuclear Science and Technology Organisation (ANSTO), either on dry-ice or at room temperature. Sterility confirmed by passages as recommended by WHO [26]. Lack of detectable HA activity, as measured by Haemagglutination assay, in allantoic fluid from 3 passages indicated complete loss of virus infectivity.

2.4. Transmission Electron Microscopy (TEM)

Irradiated A/PR8 (γ-A/PR8) samples loaded into 3 mm formvar-amorphous carbon-coated copper grids and left for 2 min. Excess solution removed by blotting. Samples stained with 2% Uranyl Acetate for 2 min, then blotted and left to dry at RT for 10 min before visualisation with FEI Tecnai G2 Spirit Transmission Electron Microscope (Adelaide Microscopy, University of Adelaide).

2.5. SDS-PAGE

Irradiated and control samples heat-treated at 85 °C for 20 min. Viral proteins separated by electrophoresis on Pre-Cast NuPAGE Novex 4–12% Bis-Tris gel (Thermo Fisher Scientific), then stained with Coomassie Brilliant Blue. Novex Sharp Pre-Stained Protein Standards (Thermo Fisher Scientific) used for MW comparison.

2.6. Mice & treatment

Six-week-old female wild-type BALB/c mice (H-2^d) supplied by Laboratory Animal Services, University of Adelaide. Mice were anaesthetized intraperitoneally (IP) with 10 μL/gram body weight ketamine anaesthetic (1% xylazine, 10% ketamine in sterile H_20), and vaccinated intranasally (IN) with one or two doses 14 days apart of γ-A/PR8. Control animals treated with PBS. 21 days post-vaccination, animals were anaesthetised, challenged IN with A/PR8 (1.6×10^2 $TCID_{50}$/mouse), and monitored for 3 weeks for clinical symptoms and weight loss. Animals were culled if they lost 20% of starting body weight.

2.7. Measurement of influenza-specific antibody responses

Blood samples collected from all mice via submandibular bleeding 20 days post-vaccination and serum levels of A/PR8-specific IgG were determined by ELISA as described previously [27]. Absorbance measured at 450/620 nm using Biotrack II plate reader, end point titres expressed as reciprocal of the last dilution where OD value ⩾ cut-off value. Cut-off value was determined as mean +(3 × S.D.) of OD values of samples from control mice.

2.8. In vitro neutralisation assay

96-well tissue-culture plates seeded with 6×10^4 MDCK cells/well. A/PR8 activated by treatment with 2 μg/mL TPCK-trypsin (Sigma-Aldrich) for 30 min at 37 °C. Heat-inactivated sera were serially diluted, mixed with A/PR8 (diluted in allantoic fluid + 4 μg/mL TPCK-trypsin) in 1:1 ratio, and incubated for 1 h at 37 °C. Mixture added to MDCK monolayers at MOI of 0.1 and incubated for 2 h at 37 °C. Then, inoculum was removed, monolayers washed with PBS and returned to incubator for 22 h in serum-free media. Monolayers washed, fixed and permeabilised with acetone/methanol (1:1 ratio) at 4 °C and incubated with polyclonal murine anti-A/PR8 sera (generated as previously described [28]) for 1 h at 4 °C. Alexa-Fluor® 488 goat anti-mouse IgG (H + L) (Life Technologies) added for 1 h at 4 °C. Nuclei stained with DAPI (1 μg/mL) for 30 min at room temperature (RT). Images acquired using a Nikon TiE inverted fluorescence microscope and analysed using NIS elements software (Tokyo, Japan).

2.9. Cytotoxic T-cell assay

Mice primed by intravenous injection of live or γ-A/PR8. 6 days later, target splenocytes from naïve mice labelled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) (0.125 mM) or CellTrace™ Far-Red DDAO-SE (2 μM, Thermo Fisher Molecular-Probes). CFSE population pulsed with Influenza A nucleoprotein peptide (GL Biochem (Shanghai) Ltd, sequence: TYQRTRALV). Target cells mixed at 1:1 ratio (CFSE/CellTrace Red) and adoptively transferred into primed mice using intravenous injection (10^7 cells/mouse). 24 h later, mice sacrificed, spleens harvested, processed to single-cell suspensions, and analysed using FACS (LSRII, BD Biosciences). Data analysed using FlowJo (Treestar Incorporated). Specific lysis as follows: lysis [%] = [1 − (%primed pulsed targets/%primed non-pulsed targets)/(%unprimed pulsed targets/%unprimed non-pulsed targets)] × 100.

2.10. Statistical analysis

Quantitative results expressed as mean ± SEM. Unpaired Student's *t*-test used for comparison of data from two separate groups, and One-way ANOVA used for comparison of data from 3 or more groups. Statistical analysis performed using GraphPad Prism 6, ver-

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