Journal of Virological Methods 207 (2014) 232–237

Contents lists available at ScienceDirect

Journal of Virological Methods

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

Short communication

Hydrogen peroxide inactivation of influenza virus preserves antigenic structure and immunogenicity

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Article history:
Received 16 May 2014
Received in revised form 1 July 2014
Accepted 4 July 2014
Available online 12 July 2014

Keywords:
Hydrogen peroxide
Influenza virus
Inactivation
Immunogenic
Immune response

A B S T R A C T

The use of live virus in the laboratory requires additional precautions, such as personnel training and special equipment, in order to limit the transmission risk. This is a requirement which not all laboratories can fulfill. In this study, a viral inactivation method is introduced using hydrogen peroxide (H_2O_2), which maintains antigenicity. Three strains of influenza viruses were inactivated and the ex vivo cellular and humoral immune responses were further analyzed, by comparing them to live viruses, in ELISpot, Multiplex and ELISA assays. In all assays, the H_2O_2 inactivated viruses displayed comparable responses to the live viruses, suggesting that the inactivated viruses still elicited immunogenic responses even though inactivation was confirmed by lack of viral replication in MDCK cells. Taken together, this study demonstrates that influenza viruses inactivated with H_2O_2 retain immunogenicity and are able to both detect humoral and elicit cellular immune responses in vitro, which could reduce the need to handle live viruses in the laboratory.

1. Introduction

Influenza virus inactivation has previously been established using several methods such as by use of heat, formalin, TritonX-100, β-propiolactone, UV/solar radiation and N_2 gas (Goldstein and Tauraso, 1970; Jonges et al., 2010; Sagripanti and Lytle, 2007; Sakudo et al., 2014). While these methods effectively inactivate the virus, they also have an effect on the antigenic structure resulting in changes in immunogenicity, which limit their use. Live influenza virus is commonly used in the laboratory to stimulate peripheral blood mononuclear cells (PBMCs) in order to study cellular immune responses (McElhaney et al., 1998, 2006; Rudenko et al., 2014). Similarly, live virus is also used to coat ELISA plates in order to measure influenza specific serum IgG/IgE and/or IgA levels from nasal secretions, which can illustrate humoral and mucosal immunity (Adar et al., 2009; Callow, 1983; Chirkova et al., 2011; Rudenko et al., 2011). This is important to be able to characterize immune responses after natural infection and vaccination efficacy after vaccination. Use of inactivated virus in these assays could have a significant impact on public health measures. It would limit the need for biosafety measures, especially in the case of highly pathogenic and novel influenza virus assessment and thereby allow not only a quicker determination of public health impact, but also increase the range in which the assessment could occur, such as in low income countries which may not have access to BSL-3 facilities. For this situation of course, it would be crucial that the inactivated virus maintained similar antigenic properties as the live virus.

H_2O_2 is a strong oxidizing agent that has been used as a disinfectant agent, since it destroys or inhibits a wide range of microorganisms (2004). Recently it was reported that H_2O_2 treatment may be an effective inactivation method for vaccine production (Amanna et al., 2012; Pinto et al., 2013). However, it has not been tested to what extent this inactivation method retains antigenic and immunological properties of the influenza virus. In this study, we evaluated the inactivation of three different influenza strains; seasonal H1N1, pandemic H1N1 (2009) and seasonal H3N2, by H_2O_2 to determine if they retained their antigenic integrity and capacity to induce immune responses.

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http://dx.doi.org/10.1016/j.jviromet.2014.07.003
0166-0934/

2. Material and methods

2.1. Cell isolation

Blood samples were collected from healthy adult volunteers as approved by the Regional Committee for Medical and Health Research Ethics South-East Norway (REC South-East, Reference number 2012/2183). Peripheral blood mononuclear cells were isolated from anticoagulated venous blood collected in ACD tubes (BD vacutainer, Plymouth, UK) by Ficoll density gradient centrifugation (Ficoll-Paque Premium 1.077, GE Healthcare, Oslo, Norway) using Sepmate 50 tubes (Stemcell Technologies, Grenoble, France) following manufacturer's instructions.

2.2. Virus propagation and titration

Three strains of influenza virus (A/California/07/2009(H1N1) pdm09, A/New Caledonia/20/1999(H1N1) and A/Brisbane/10/2007 (H3N2) (provided by the WHO Collaborating Centre for Reference and Research on Influenza, National Institute for Medical Research, London UK)) were propagated by inoculating 100 μl allantoic fluid into the allantoic cavity of 9–11 days old specific antibody negative embryonated chicken eggs (Nortura Samvirkekylling, Våler i Solør, Norway). Presence of hemagglutinating agent was determined by the hemagglutination assay using 1% chicken erythrocytes (World Health Organisation, 2002). Propagated virus was titrated on Madin-Darby canine kidney (MDCK) cells (kindly provided by Dr. Sharon M. Brooks, AHVLA, UK). MDCK cells were cultured in MEM Minimum Essential Medium (Gibco by Life Technologies, Paisley, UK) with 10% FBS (Gibco by Life Technologies), 0.1 mM MEM NEAA (Gibco by Life Technologies), 25 mM Hepes Buffer (Lonza BioWhittaker, Walkersville, MD, USA), 2 mM L-glutamin (Lonza BioWhittaker) and 100 U/ml penicillin/streptomycin (Lonza BioWhittaker) in a 96-wells plate for 24 h. All culture incubations were performed at 37 °C, 5% CO_2 humidified incubator. Subsequently, cells were washed twice with PBS buffer (PBS de Boer ($Na_2HPO_4 \times 2H_2O$ 1.34 g/l, $NaH_2PO_4 \times H_2O$ 0.34 g/l, NaCl 8.5 g/l, pH: 7.2 ± 0.1)) and inoculated with a ten-fold dilution of propagated virus. Virus was diluted in media described above but without 10% FBS and supplemented with 1 μg/ml TPCK-treated trypsin (Sigma-Aldrich, St. Louis, MO, USA). 48 h post inoculation, cells were observed for cytopathic effect (CPE) and 50 μl cell media was removed from each well and tested by hemaglutination assay as described above. Virus titer was calculated by using the Karber formula (Kärber, 1931).

2.3. Virus inactivation

The viruses were inactivated in a 1% H_2O_2 or 3% H_2O_2 (Sigma-Aldrich) solution (final concentration) in PBS (Gibco by Life Technologies) for 2 h at room temperature (or 4 h if stated), then diluted in AIM-V media (Gibco by Life Technologies) or PBS (Gibco by Life Technologies) and used for further experiments.

2.4. Innocuity testing in MDCK cells

Assessment of CPE: 30,000 MDCK cells per well were plated in triplicate in 48 well plates (Corning Costar, Hazebrouck Cedex, France) and allowed to attach overnight at 37 °C with 5% CO_2. The following morning, when the cells were 60–80% confluent, they were inoculated with either live, 1% H_2O_2 or 3% H_2O_2 inactivated viruses at 12.15 HAU in 150 μl serum free media for 1 h at 37 °C. After one hour, supernatants were removed and the cells were washed 3× with PBS, then complete media was added and the cells were incubated. After 48 h, media was removed and cells were stained with crystal violet (Sigma-Aldrich) as described previously (Dembinski et al., 2010). Images were taken using a CTL 6 Ultra V analyzer (Bonn, Germany).

PCR Replication Analysis: MDCK cells were plated in duplicate in T25 tissue culture flasks (Nunc, Thermo Scientific, Roskilde, Denmark) and allowed to adhere at 37 °C, with 5% CO_2. When the cell layer was 70–80% confluent, the cells were inoculated with either live, 1% H_2O_2 or 3% H_2O_2 inactivated viruses at 12.15 HAU or a media blank, 1% H_2O_2 blank or 3% H_2O_2 blank in 1 ml serum free media for 1 h at 37 °C. After 1 h, supernatants were removed and the cells were washed 2× with PBS, and complete media was added. Supernatants were collected on day 0, 1, 2, 3, 6, 7 and 8 post inoculation. Additionally, on days 3, 7 and 8, the cells were passaged and 50% of the cells were lysed. On day 7, 1 ml of the supernatants were passaged to new MDCK cells, in which supernatants and cell lysates were harvested on day 8.

RNA was purified using the Roche MagNA Pure LC Total Nucleic Acid Isolation kit, according to manufacturer's instructions, using 140 μl of input material and 60 μl of elution volume. The presence of influenza A matrix gene RNA was determined using a semi-quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay, developed by the U.S Centers for Disease Control and Prevention and published on the World Health Organization web site (CDC, 2009).

2.5. IFN gamma ELISpots

Mabtech IFN-γ pre-coated 96 well plates were used according to the manufacturer's instructions. 200,000 PBMCs were added per well in AIM-V media. Antigenic stimulants and concentration used were as follows; negative controls (AIM-V media, 1% H_2O_2 or 3% H_2O_2 in PBS), positive control (ConA (Sigma-Aldrich) 5 μg/ml), live virus (A/California/07/2009(H1N1)pdm09, A/New Caledonia/20/1999(H1N1) and A/Brisbane/10/2007(H3N2) 15 HAU (unless otherwise stated)), 1% H_2O_2 or 3% H_2O_2 inactivated virus (A/California/07/2009(H1N1)pdm09, A/New Caledonia/20/1999(H1N1) and A/Brisbane/10/2007(H3N2) 15 HAU (unless otherwise stated)).

Plates were incubated overnight in a 37 °C, 5% CO_2 humidified incubator and developed the following day. The plates were then read using a CTL S 6 Ultra V analyzer, and results were plotted using GraphPad software displaying the mean and standard deviation.

2.6. Multiplex cytokine detection

100 μl of the stimulated PBMC supernatants was removed from the overnight incubated IFNg ELISpot plates and stored at −20 °C. Using the Bio-Plex Pro human cytokine Th1/Th2 panel kit (with the addition of Th17) (Bio-Rad, Hercules, CA, USA), 50 μl of this was analyzed on a Bio-Plex 200 system according to the manufacturer's instructions. Data analyses were performed using the Bio-Plex manager system and the results were plotted using GraphPad software displaying the mean and standard deviation.

2.7. ELISA

IgG antibodies in serum to either live or inactivated virus were measured by enzyme-linked immunosorbent assay (ELISA). Briefly, 100 μl of the live or 3% H_2O_2 inactivated virus, diluted in PBS, at concentrations of 5, 7.5 or 10 μg/ml (where 150 HAU ≈ 1 μg) were added per well to 96 well plates (MaxiSorb, Nunc, Roskilde, Denmark) and allowed to bind for a minimum of 2 days at 4 °C. Non-specific protein-binding sites were blocked with 5% dry skimmed milk (Oxoid, Basingstoke, UK) in PBS. Two-fold serial dilutions of a pooled sera standard (from donors as listed in Section 2.1) were applied in duplicate. The plates were incubated overnight at 4 °C and subsequently incubated for 1 h at room temperature