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Efficient influenza B virus propagation due to deficient interferon-induced antiviral activity in MDCK cells

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ABSTRACT

Influenza B virus infections are mainly restricted to humans, which is partially caused by the inability of influenza B virus NS1 protein to counteract the innate immune response of other species. However, for cell culture-based influenza vaccine production non-human cells, such as Madin–Darby canine kidney (MDCK) cells, are commonly used. Therefore, the impact of cellular pathogen defence mechanisms on influenza B virus propagation in MDCK cells was analysed in this study. Activation of the cellular antiviral defence by interferon stimulation slowed down influenza B virus replication at early time points but after 48 h the same virus titres were reached in stimulated and control cells. Furthermore, suppression of the antiviral host defence by transient expression of a viral antagonist, the rabies virus phosphoprotein, could not increase influenza B virus replication. Finally, canine Myxovirus resistance (Mx) proteins showed no antiviral activity in an influenza B virus-specific minireplicon assay in contrast to the murine Mx1 protein. Taken together, these results indicate that an insufficient antiviral defence in MDCK cells promotes efficient influenza B virus replication favouring the use of MDCK cells in influenza vaccine production.

1. Introduction

Influenza A and B viruses represent a high burden for human health. These viruses cause an acute respiratory disease resulting annually in three to five million cases of severe illness and 250,000 to 500,000 deaths worldwide (http://www.who.int/topics/influenza/en/). The best protection against influenza is provided by vaccination. Currently the recommended trivalent vaccines contain two influenza A strains (an H1N1 subtype and an H3N2 subtype) in combination with one influenza B virus, which represent the most prevalent circulating strains identified by the WHO Global Influenza Surveillance Network.

The majority of these influenza vaccines is still produced in embryonated hen's eggs. This production process is well-established, but it is certainly neither flexible nor scalable enough to satisfy increasing demands in a pandemic situation. To overcome these limitations vaccine manufacturers are currently establishing cell culture-based production processes. Therefore, continuous mammalian cell lines are propagated in bioreactors, a scalable and controlled environment [1]. Besides the investigation of cultivation strategies, understanding of virus–host cell interactions is crucial for the optimisation of cell culture-based vaccine manufacturing processes.

Madin–Darby canine kidney (MDCK) cells are well-known to be highly permissive for infections with different influenza virus strains [2], and MDCK cell culture-derived influenza vaccines were already approved by the European Medicines Agency [3]. However, MDCK cells are able to sense virus infections by so-called pattern recognition receptors (PRRs) leading to the induction and secretion of interferon (IFN), which in turn activates the cellular pathogen defence [4,5]. Among several hundreds of IFN-stimulated genes (ISGs) some possess an antiviral activity, such as myxovirus resistance (Mx) proteins. Mx proteins are dynamin-like large GTPases, which confer resistance against orthomyxoviruses in mice by a still unknown mechanism [6]. Another antiviral protein is ISG15, a small ubiquitin-like molecule, which can be conjugated to over 100 known target proteins. The knock-out of components of this ISG15 conjugation system (ISGylation) in mice results in increased susceptibility to influenza A and B virus infections [7,8]. Furthermore, IFN stimulates the expression of its own transcriptional activator, IFN regulatory factor 7 (IRF7), creating a positive feedback mechanism [5].

In previous studies we analysed the influence of cellular innate immune response on influenza A virus yields in MDCK cells. Intracellular signal transduction pathways that are related to virus-sensing and subsequent activation of cellular pathogen defence mechanisms were differentially induced by diverse influenza A

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virus strains [9,10]. Yet, neither the inhibition nor the stimulation of these antiviral signalling pathways had an impact on maximum influenza A virus titres obtained in MDCK cell cultures. Therefore, cellular innate immune response has only a minor impact on influenza A virus replication in MDCK cell culture-based vaccine production [10].

Influenza B viruses, however, differ from influenza A viruses with regard to their countermeasures against innate immunity [11]. The non-structural protein 1 (NS1) of influenza B viruses binds to ISG15 and inhibits the ISGylation. This antagonistic mechanism is effective in humans but not in canine cells like MDCK [12]. Indeed, this species-specific antagonism was suggested to play a role in the limited host range of influenza B viruses whose infections are largely restricted to humans [13].

In this report we studied the influence of the cellular innate immune response on influenza B virus propagation in MDCK cells. Stimulation of the innate immune response was done by IFN treatment prior to infection to analyse whether an activated antiviral state reduces virus titres in MDCK cells. Furthermore, antiviral signalling was suppressed to examine if the inhibition of innate immunity could enhance influenza B virus yields in cell culture-based influenza vaccine production.

2. Materials and methods

2.1. Cells and viruses

MDCK cells (ECACC No. 84121903) were cultivated and infected with influenza virus according to Genzel et al. [14]. The influenza strain B/Malaysia/2506/2004 (National Institute for Biological Standards and Control) belongs to the B/Victoria-lineage and was included in the influenza vaccine used in the southern hemisphere for two seasons (2006, 2007), and during two northern hemisphere seasons (2006/2007, 2007/2008). Virus stocks were prepared in MDCK cells. The influenza virus A/PuertoRico/8/34-delNS1 (AVIR Green Hills Biotechnology) was grown in Vero cells (ECACC No. 88020401) cultured under the same conditions as MDCK cells. Multiplicity of infection (MOI) was calculated based on viable cell number at the time of infection and active virus titre ($TCID_{50}$) of virus stocks. Human embryonic kidney cells (HEK 293T) were maintained in Dulbecco's modified Eagle medium (DMEM high glucose; # F-0455; Biochrom) supplemented with 10% FCS, glutamine and antibiotics.

2.2. Transfection

For transient transfection of MDCK cells the Microporator (Digital Bio) was used. Electroporation was done in 100 μL microporation tips using one pulse of 1700 V for 20 ms with 8 μg DNA per 3×10^6 cells. 0.3×10^6 cells were seeded per well of 24-well plates. Cells were infected 24 h post transfection.

The expression vector pCR3 containing the phosphoprotein of rabies virus (rP) was provided by the group of Karl–Klaus Conzelmann (Max von Pettenkofer Institute, Ludwig-Maximilians-University Munich, Germany).

2.3. Stimulation with conditioned medium

Conditioned medium (CM) of MDCK cells infected with influenza virus A/PuertoRico/8/34-delNS1 at an MOI of 5 (without trypsin) was prepared as described by Seitz et al. [10]. Medium of mock infected cells served as a control. For the stimulation, 1×10^6 MDCK cells in 35 mm dishes were washed twice with PBS and 1.5 mL of CM was used. Stimulation was done for 5 h, and subsequently 0.5 mL medium was added containing influenza virus B/Malaysia/2506/2004 (MOI of 0.025), trypsin and gentamicin (final concentration 0.1 mg/mL, Gibco). At indicated time points virus load of the supernatant was determined by hemagglutination assay.

Table 1
Primers used for qRT-PCR.

Primer	Nucleotide sequence
IFN-β forward	5′-CCAGTTCCAGAAGGAGGACA-3′
IFN-β reverse	5′-TGTCCCAGGTGAAGTTTTCC-3′
Mx1 forward	5′-GAATCCTGTACCCAATCATGTG-3′
Mx1 reverse	5′-TACCTTCTCCTCATATTGGCT-3′
ISG15 forward	5′-TCTGTGCCCCTGGAGGACTTGA-3′
ISG15 reverse	5′-TGCTGCTTCAGCTCTGATGCCA-3′
18s forward[a]	5′-CGGACAGGATTGACAGATTG-3′
18s reverse[a]	5′-CAAATCGCTCCACCAACTAA-3′

[a] http://medgen.ugent.be/rtprimerdb, Primer ID 3879.

2.4. Hemagglutination assay

Hemagglutination activity was determined as previously described [15]. Titres are reported as log HA units per assay volume (log HA/100 μL).

2.5. RNA extraction and reverse transcription

Total RNA isolation was done using the NucleoSpin RNA II Kit (Macherey–Nagel) according to manufacturer's instructions.

RevertAid M-MuLV-RT (Fermentas) was applied for reverse transcription of cellular mRNA. 1 μg total RNA was transcribed with an Oligo(dT)-primer according to manufacturer's reaction conditions.

2.6. qRT-PCR

Real-time quantitative reverse-transcription PCR (qRT-PCR) was performed on a Rotor-Gene Q cycler (Qiagen) using the Rotor-Gene SYBR Green PCR Kit (Qiagen). Sequences of primers used are summarised in Table 1. 0.4 μl of reverse transcription reaction were analysed in a final reaction volume of 15 μl. Reaction set up and thermal cycling parameters were taken from the technical data sheet of the master mix. Expression of IFN-β, ISG15 and Mx1 was calculated by the $2^{-\Delta\Delta ct}$-method, using 18s-rRNA for data normalisation and uninfected cells as calibrator.

2.7. Western blots

Protein sample preparation and Western blots were done according to Heynisch et al. [9] using antibodies against MxA (M143, [16]), and as a loading control ERK2 (SC-153, Santa Cruz Biotechnology).

2.8. Minireplicon assay

To analyse inhibitory properties of selected Mx proteins on reconstituted viral polymerase activity, a minireplicon assay was performed. HEK 293T cells seeded on 12-well plates were transiently transfected with 1 μg total DNA using 3.2 μl Nanofectin (PAA Laboratories) following the manufacturer's protocol. 10 ng of pCAGGS expression plasmids encoding the viral polymerase subunits PB2, PB1 and PA and 50 ng NP derived from influenza virus B/Yamagata/73 [17] were co-transfected with 25 ng of the influenza B minigenome expression plasmid pPolI-lucRT_B encoding *firefly* luciferase flanked by the non-coding region of segment 8 of B/Yamagata/73 [18] as well as 10 ng pRL-SV40-Rluc encoding *renilla* luciferase for normalization of the transfection efficiency.