

# Leukocyte Interferon- $\alpha$ -n3 Inhibits *Influenza* A Viral Replication in Human Alveolar Epithelial A549 Cells

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## Abstract

The emergence of the drug-resistant *Influenza* A strains including the antiviral resistant 2009 pandemic H1N1 (pH1N1) highlighted this urgent need to develop new antiviral strategies for protecting against infection of *Influenza* virus. Type I interferon including interferon alpha and beta (IFN- $\alpha/\beta$ ) produced by host cells in response to the presence of pathogens, plays a critical role in viral pathogen clearance during infection. In this study we determined the effect of Alferon N Injection® (a natural interferon alpha product derived from human leukocytes, IFN- $\alpha$ -n3) on the *Influenza* A virus including pH1N1 virus isolated from pigs, Avian H9N2 and Swine H3N2 viruses in human alveolar epithelial A549 cells. The results showed that IFN- $\alpha$ -n3 is able to inhibit replication of these three *Influenza* A viruses in human alveolar epithelial cells, indicating that IFN- $\alpha$ -n3 may be useful in treating *Influenza* clinically.

**Keywords:** Interferon- $\alpha$ -n3; Inhibition; *Influenza* A virus; Replication

## Introduction

Although the 2009 flu pandemic did not cause the high mortality rates as predicated in June, 2009 when the world health organization (WHO) declared the first pandemic in 41 years, the outbreak reminded us that *Influenza* remains a major infectious disease and public health challenge. This outbreak reinforced the problems of vaccine production capacity and supply in response to a pandemic. For example, no pandemic H1N1 vaccine was available in most developing countries even more than 8 months after the WHO declared the pandemic [1]. Although the current seasonal *Influenza* and pandemic H1N1 vaccines have proven to be effective in protecting against infection, the average time frame to manufacture an *Influenza* vaccine is at least 6 months, which is not sufficient to meet the demands of a pandemic in a timely manner. Moreover, safety concerns still exist for people with egg allergies because the viruses for *Influenza* vaccines are commonly derived from chicken egg-based cultures. Besides vaccination, antiviral treatment is an important strategy in helping to control and prevent an *Influenza* pandemic. Two types of antivirals, M2 ion channel inhibitors (Amantadine and Rimantadine) and neuraminidase inhibitors (Oseltamivir and Zanamivir), are commercially available in the United States and have been shown to reduce the severity and durations of flu-illness symptoms. Both antiviral treatments have side effects including: lightheadedness, anxiety, chills, nausea, vomiting, loss of appetite and trouble breathing. It is important to note that the 2009 pandemic H1N1 virus (pH1N1) is resistant to M2 inhibitors and an NA-inhibitor resistant pH1N1 strain has also emerged in patients [2]. The emergence of an antiviral-resistant 2009 pH1N1 virus highlighted the urgent need to explore novel antiviral strategies for controlling and preventing *Influenza* infection.

No one can predict which subtype will cause the next pandemic. The highly pathogenic H5N1 Avian *Influenza* virus is able to directly infect humans and have caused more than 60% mortality in infected humans since the first case was reported in Hong Kong, China in 1997 [3,4]. Therefore, the highly pathogenic H5N1 Avian *Influenza* virus has been thought to be the most likely of all candidates to cause the next pandemic. The H9N2 Avian *Influenza* virus has been considered to be another candidate causing the next pandemic because infections with this subtype of virus in humans have been reported [5,6] and normally humans do not have immunity against this subtype of virus. Recently, more than 10 human cases infected with triple reassortant H3N2 Swine *Influenza* virus containing the M gene from the pH1N1 virus were reported in the USA [7]. The common feature is that these viruses do not have the ability of human-to-human transmission. Nevertheless, if these viruses gained this ability, they will be serious threats for public health. Therefore, it is necessary to prepare vaccines and antivirals for the next pandemic.

Type I interferon including interferon alpha and beta (IFN- $\alpha/\beta$ ) produced by host cells in response to the presence of pathogens, plays a critical role in pathogen clearance during infection [8]. A recent study showed that the 2009 pH1N1 is highly sensitive to the antiviral actions of interferons including type I (IFN- $\alpha/\beta$ ) and type III (IFN- $\lambda$ 3) [9]. Alferon N injection® (IFN- $\alpha$ -n3), produced by Hemispherx Biopharma, Inc, is a natural interferon alpha product derived from human leukocytes which contains at least 7 major alpha interferon species. It has been approved by the US food and drug administration in the treatment of refractory or recurrent *Condylomata acuminata*. We have showed that IFN- $\alpha$ -n3 was not toxic even at a concentration of 10,000 IU/ml for both MDCK and human alveolar epithelial A549 cells (data not shown) using the ToxiLight™ bioassay kit (Lonza). In this report we investigated whether IFN- $\alpha$ -n3 is able to inhibit

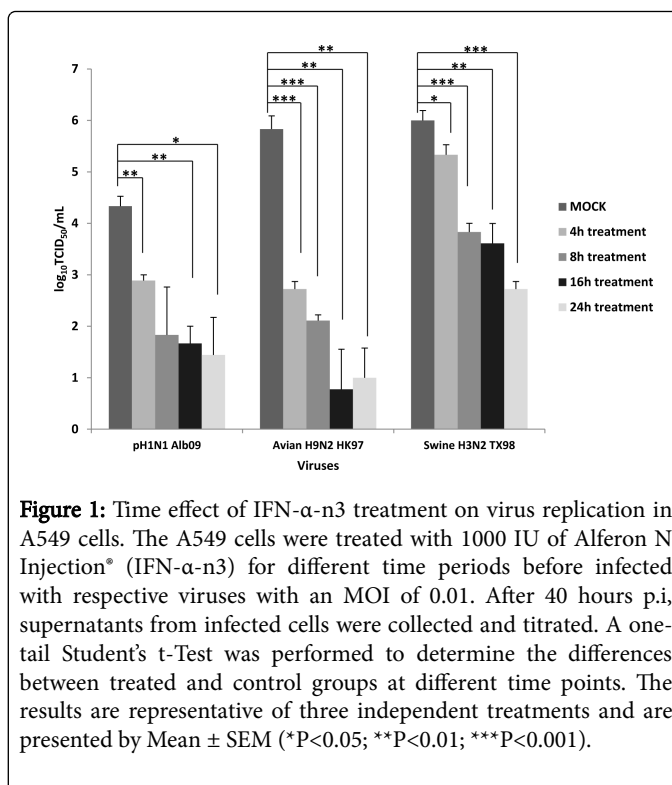
*Influenza A* viruses in vitro including the pH1N1, Avian H9N2 and Swine H3N2 viruses.

## The Study

To examine the effect of IFN- $\alpha$ -n3 on the pH1N1, Avian H9N2 and Swine H3N2 virus replication, we studied different concentrations of IFN- $\alpha$ -n3 treating human alveolar epithelial A549 cells (ATCC, CCL-185) which were then infected with respective viruses at the indicated multiplicity of infection (MOI), respectively. A Swine-origin pH1N1 (A/Swine/Alberta/25/2009, Alb09) virus was kindly provided by Dr. Webby at St. Jude Children's Research Hospital and used in this study which was isolated from the Canadian pigs and the genome of this virus exhibited 99.6%-100% identity at nucleotide acid level with that of the human isolate (A/CA/04/09, CA09). We have shown that both Alb09 and CA09 pH1N1 viruses are highly pathogenic and transmissible in pigs [10]. An Avian H9N2 A/Quail/Hong Kong/G1/1997 (HK97) generated by reverse genetics [11] and an H3N2 triple reassortant A/Swine/Texas/4199-2/98 virus (TX98), which was obtained from the repository at St. Jude Children's Research Hospital, Memphis, Tennessee were utilized in this study; the HK97 virus is genetically and antigenically similar to an H9N2 virus which infected humans in 1999 [6].

The confluent human alveolar epithelial A549 cells in a 48-well plate were treated with different units of IFN- $\alpha$ -n3 (10,000 IU/well, 1,000 IU/well, 100 IU/well, 10 IU/well) in a volume of 20  $\mu$ l minimum essential medium (MEM), or mock-treated with 20 $\mu$ l MEM for 4 hours, then directly infected with the pH1N1 Alb09, H9N2 HK97 or H3N2 TX98 virus at MOI of 0.01 in 500  $\mu$ l of infecting medium containing 0.3% bovine albumin and 1  $\mu$ g/ml TPKC-treated trypsin without removing the treated IFN- $\alpha$ -n3 from media, respectively. The inoculated viruses were kept in the cell media throughout the experiment. The supernatants were collected at 48 hours post infection (p.i) and titrated in MDCK cells in a 96-well plate as described previously [12]. Plates were evaluated for cytopathic effects after 48 hours p.i. At 72 hours p.i the plate was fixed with 4% phosphate-buffered formaldehyde and immunocytochemically stained with a monoclonal antibody to *Influenza A* nucleoprotein. The TCID<sub>50</sub> per milliliter was calculated by the method of Reed and Muench [13]. The results showed that all tested doses of IFN- $\alpha$ -n3 could not efficiently inhibit Avian H9N2 HK97 and Swine H3N2 TX98 virus replication in human alveolar epithelial A549 cells prior to 4 hours treatment. Moreover, high doses (1,000 IU and 10,000 IU) of IFN- $\alpha$ -n3 were able to significantly inhibit pH1N1 Alb09 replication prior to 4 hours treatment (data not shown).

To determine an optimal time for treatment with the IFN- $\alpha$ -n3 in human alveolar epithelial A549 cells, we treated cells with 1000 IU of IFN- $\alpha$ -n3 or mock-treated for 4, 8, 16 and 24 hours, then infected them with different viruses at an MOI of 0.01. Supernatants from infected cells were collected at 40 hours p.i and titrated. The results showed that 16 hours of treatment prior to infection are needed to efficiently inhibit replication of these 3 viruses in human alveolar epithelial A549 cells (Figure 1).



**Figure 1:** Time effect of IFN- $\alpha$ -n3 treatment on virus replication in A549 cells. The A549 cells were treated with 1000 IU of Alferon N Injection<sup>®</sup> (IFN- $\alpha$ -n3) for different time periods before infected with respective viruses with an MOI of 0.01. After 40 hours p.i, supernatants from infected cells were collected and titrated. A one-tail Student's t-Test was performed to determine the differences between treated and control groups at different time points. The results are representative of three independent treatments and are presented by Mean  $\pm$  SEM (\* $P$ <0.05; \*\* $P$ <0.01; \*\*\* $P$ <0.001).

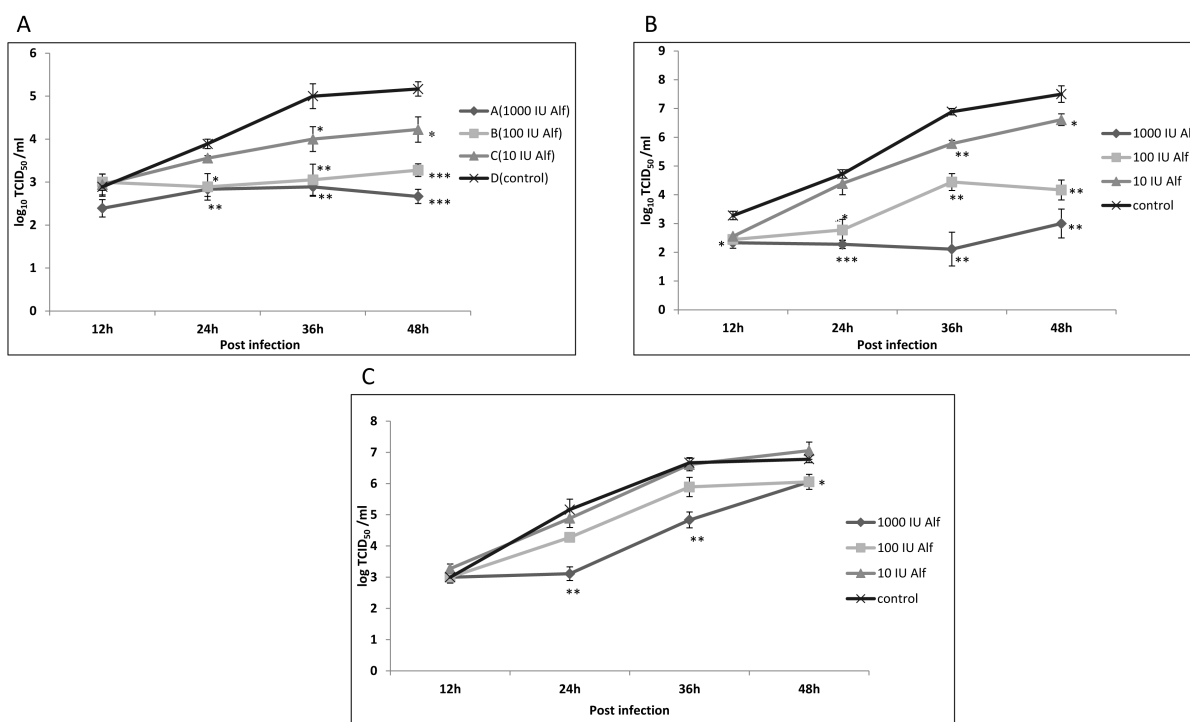
To determine the minimal dose for efficient inhibition of virus replication, we treated cells with different units of IFN- $\alpha$ -n3 (1,000 IU, 100 IU or 10 IU) for 16 hours prior to infection of respective virus at an MOI of 0.01 or MEM as a control. Results showed that 100 IU of IFN- $\alpha$ -n3 is sufficient to inhibit replication of the pH1N1 Alb09 and Avian H9N2 HK97 viruses (Figures 2A and 2B), but it is not enough to inhibit replication of the Swine H3N2 TX98 virus (Figure 2C).

## Conclusion and Discussion

Vaccination is the principal means of prevention and control of clinical *Influenza* infections and antiviral drugs are used as an adjunct to vaccination for the control of symptoms of *Influenza*. However, the rapidly evolving nature of the *Influenza A* virus results in challenges in producing effective vaccines and antivirals for the control and prevention of both epidemic and pandemic. The 2009 *Influenza* pandemic was caused by a reassortant Swine H1N1 virus between North American triple reassortant and Eurasian Swine *Influenza* viruses [14]. The pH1N1 virus is antigenically significantly distinct from seasonal *Influenza* viruses; therefore, the majority of human population lacked immunity against this virus. Fortunately, this pandemic was relatively mild and overall in most countries mortality was lower than predicted. Lessons that we learned from the 2009 *Influenza* pandemic highlighted the urgent need for finding new drugs to control or prevent *Influenza* virus pandemics, especially those that result in the emergence of drug-resistant pH1N1 virus. In this study we evaluated the effect of IFN- $\alpha$ -n3 on the pH1N1 and other 2 *Influenza A* viruses including Avian H9N2 HK97 and Swine H3N2 TX98 viruses. Both Avian H9N2 and Swine H3N2 viruses have been documented to infect humans [6,7]. Our results demonstrated that 100 IU of IFN- $\alpha$ -n3, a natural leukocyte interferon alpha product, can efficiently inhibit replication of 2009 pH1N1 and Avian H9N2 viruses in human A549 cells; however, 16 hours of incubation is needed.

Interestingly, similar amount of IFN- $\alpha$ -n3 cannot efficiently inhibit replication of the Swine H3N2 virus in human alveolar epithelial A549 cells. Further studies need to investigate why the Swine H3N2 TX98 is not as sensitive to the IFN- $\alpha$ -n3 as the other 2 viruses. A longer treatment with IFN- $\alpha$ -n3 required for efficient inhibition of *Influenza* virus replication in human alveolar epithelial A549 cells suggests that it can be used efficiently for pre-exposure prophylaxis of *Influenza*

infection. Recently, one study showed that low-dose type I interferon is effective against H5N1 and pandemic H1N1 viruses in vitro and in a mouse model [15]. Furthermore, type I interferon has been used clinically in the treatment of hepatitis B and C. All these results indicate that type I interferon could be a good candidate for the antiviral treatment of *Influenza* viral infection.



**Figure 2:** Inhibition of replication of (A) the 2009 pH1N1 Alb09, (B) Avian H9N2 HK97 and (C) Swine H3N2 TX98 viruses by different units of Alferon N Injection® (IFN- $\alpha$ -n3) at indicated time points in A549 cells. The cells were treated with IFN- $\alpha$ -n3 for 16 hours prior to infection with respective viruses at an MOI of 0.01. A one-tail Student's t-Test was performed to determine the differences between treated and control groups at different time points. The results are representative of three independent treatments and are presented by Mean  $\pm$  SEM (\*P<0.05; \*\*P<0.01; \*\*\*P<0.001).

Completely different antiviral mechanisms of action are used by type I Interferon (IFN- $\alpha/\beta$ ) compared to the currently FDA approved anti-*Influenza* drugs. IFN- $\alpha/\beta$  up regulates the expression of numerous IFN-stimulated antiviral genes, which include protein kinase R (PKR) and 2',5'-oligoadenylate synthetase (OAS) that are activated by the double-stranded RNA [16]. The activated PKR phosphorylates the eukaryotic initiation factor eIF2 $\alpha$ , thereby inhibiting viral mRNA translation and viral replication [17]. The OAS degrades cellular and viral single-stranded RNA, leading to inhibition of cellular and viral protein synthesis [16]. In contrast to the PKR and OAS, another IFN ( $\alpha/\beta$ ) upregulated gene product, the Mx protein, directly interacts with viral components to trap and sort them from cellular compartments where they are unavailable to generate new virus particles [15,18]. Although a recent study revealed that the pH1N1 is partially resistant to the antiviral activity of IFN $\alpha$  and other IFN $\alpha$  subtypes [19], our current and former studies [9,15] clearly demonstrated that type I interferons are able to inhibit the *Influenza* A viruses including the 2009 pH1N1 and Avian H9N2 efficiently in a low dose *in vitro* or *in vivo*. Further studies are needed to determine the efficacy of IFN- $\alpha$ -n3 on the inhibition of the pH1N1 and Swine

*Influenza* viruses in the pig model, or of the Avian *Influenza* virus in the chicken model.

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