Neuraminidase Activity of Influenza Virus Strains that Differ in the Ability to Cause Disease

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Abstract

Influenza viruses are classified based on their surface glycoproteins: hemagglutinin and neuraminidase. Hemagglutinin (HA) is responsible for binding to the host cell, while neuraminidase (NA) facilitates escape of newly formed virus particles. These studies determined the NA activity of three subtypes of influenza A virus that differ in their ability to cause disease in mice: influenza A/NWS/33 (H1N1), influenza A/ Victoria/3/75 (H3N2), and influenza A/Duck/MN/1525/81 (H5N1). Chemiluminescent quantitation of NA activity in equal amounts of each virus was determined in three replicate experiments. Results indicate that N1 virus subtypes have higher NA activity than do N2 subtypes. In addition, the NA activity of each virus was tested in the presence of the NA inhibitor oseltamivir. Effective antiviral concentrations of oseltamivir for each virus were EC_{50} = 0.51, 0.19, and 0.70 nM for NWS, Victoria, and Duck viruses, respectively. These results do not support the hypothesis that NA activity alone determines the ability of the virus to cause disease. However, these data do suggest a correlation between NA activity and virus resistance to oseltamivir.

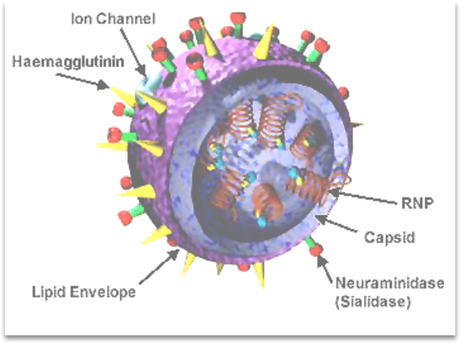

Figure 1: A model of the influenza virus [5].

Introduction

Neuraminidase activity is critical for the virus to release itself from infected cells [1]. This in turn will cause subsequent virus spread. NA has also been proposed to aid in the entry of the virus into target cells [2]. This occurs during the initial stages of infection. The roles of NA in influenza are important for its success and ability to cause disease. A focus of current chemotherapeutic treatment regimens involve inhibiting the activity of the NA enzyme. These antiviral drugs are called neuraminidase inhibitors. Oseltamivir, zanamivir, and peramivir are used and studied today. Influenza has shown an incredible increase of resistance to oseltamivir. In the 2007 - 2008 winter, the virus tested positive for resistance 11% of the time from throat swabs of patients with the most common influenza subtype [3]. Since then, the resistance of the virus has been increasing.

Methods

Virus subtypes and the NA inhibitor, Oseltamivir, were provided by the Institute for Antiviral Research. The virus stocks used were Influenza A/NWS/33 (H1N1), influenza A/Victoria/3/75 (H3N2), and influenza A/Duck/MN/1525/81 (H5N1). In each test, virus subtypes were used at equal titers and run in triplicate.

The effect of oseltamivir on viral neuraminidase activity was performed using a commercially available kit (NA-Star® Influenza Neuraminidase Inhibitor Resistance Detection Kit, Applied Biosystems, Foster City, CA) in 96-well solid white microplates following the manufacturer's instructions [4,6]. Oseltamivir in half-log dilution increments was incubated with virus (as the source of neuraminidase). The amount of virus in each microwell was approximately 500 cell culture infectious doses ($CCID_{50}$). Plates were pre-incubated for 20 minutes at 37°C prior to addition of chemiluminescent substrate. Following addition of substrate plates were incubated for 30 minutes at 37°C. The neuraminidase activity was evaluated using a Centro LB 960 luminometer (Berthold Technologies) for 0.5 seconds immediately after addition of NA-Star® accelerator solution. Fifty percent virus-inhibitory concentrations (EC_{50} values) of viral neuraminidase activity were determined by plotting percent chemiluminescent counts versus $\log_{10}$ of oseltamivir concentration. NA activity of each virus subtype was determined by completing the assay using only buffer.

Antiviral activity of oseltamivir carboxylate, the active form of oseltamivir, was determined in Madin-Darby canine kidney (MDCK) cells. The cells were grown in MEM supplemented with 5% FBS. The assays were completed in 96-well microplates infected with approximately 50 $CCID_{50}$ of virus. Microplates were visually examined after 3 days of infection and then treated for 1.5 hours with neutral red (0.011% final concentration) to quantify the virus-induced cytopathic effects (CPE). Excess dye was rinsed from cells with PBS. The absorbed dye was eluted from the cells by addition of 0.1 ml of 50% Sorensen's citrate buffer/50% ethanol to each well. Optical density (OD) measurements were completed on the microtiter plates at 560 nm. OD readings were converted to percent of uninfected control using an Excel spread sheet developed for this purpose. EC_{50} values were determined by plotting percent CPE versus $\log_{10}$ of inhibitor concentration.

Results

The virus titers were 1 x $10^{4.8}$, 1 x $10^{6.3}$, and 1 x $10^{6.4}$ PFU/ml for NWS, Victoria, and Duck viruses respectively. Virus titers were equalized using dilutions. The dilutions used were 1:5, 1:90, and 1:90 for NWS, Victoria, and Duck viruses, respectively.

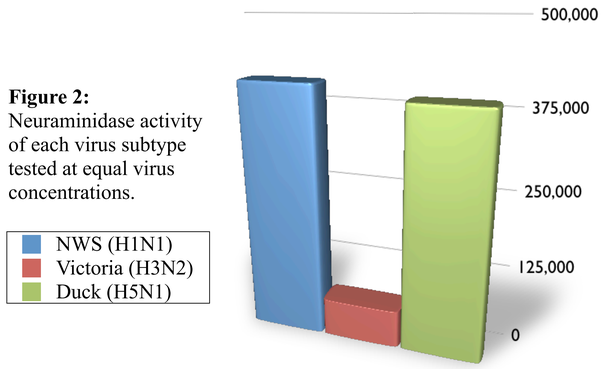

Figure 2: Neuraminidase activity of each virus subtype tested at equal virus concentrations.

Shown by Figure 2, NA activity levels at equal concentrations of virus indicate that the N1 subtype has a higher NA activity than does the N2 subtype. Additional testing of several other influenza virus subtypes have corroborated these data.

Figure 3 shows the dose-response curve of each of the viruses in the presence of oseltamivir. Effective concentration values of oseltamivir for each virus subtype were EC_{50} = 0.51, 0.19, and 0.70 nM for NWS, Victoria, and Duck viruses, respectively. These data indicate that the Duck virus is more resistant to oseltamivir than is the NWS or the Victoria viruses. The NWS virus is more resistant than is the Victoria virus. A virus expressing resistance is one that requires a higher concentration of oseltamivir to inhibit the NA activity. These data also indicate that a higher concentration of oseltamivir than was used in these assays would be required for complete inhibition of NA activity for the Duck virus.

Results shown in Figure 4 are the mean and standard deviation of four replicates of *in vitro* testing in MDCK cells. The visual observation indicates that a higher concentration of oseltamivir is required to inhibit the Duck virus compared to the NWS virus and the Victoria virus.

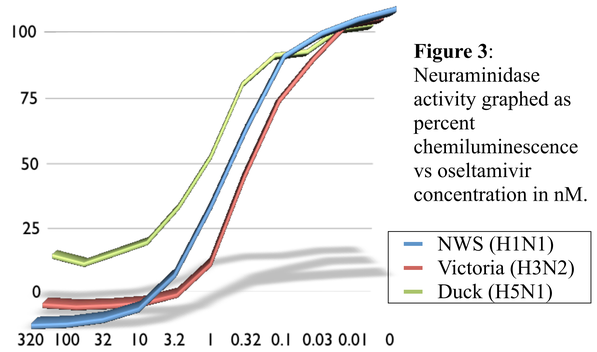

Figure 3: Neuraminidase activity graphed as percent chemiluminescence vs oseltamivir concentration in nM.

Effective Concentration of Oseltamivir						
Influenza Subtype	Visual			Neutral Red		
	EC_{50} (μM)	**CC_{50}** (μM)	**SI**	**EC_{50}** (μM)	**CC_{50}** (μM)	**SI**
NWS (H1N1)	0.141±0.045	>32	>256±110	17.2±13.6	>32	>5.28±7.72
Victoria (H3N2)	0.090±0.038	>32	>407±128	10.76±11.5	>32	>4.23±4.17
Duck (H5N1)	4.67±1.12	>32	>7.2±1.99	8.80±3.17	>32	>4.3±2.40

EC_{50} - Effective concentrations **CC_{50}** - Cell cytotoxicity **SI** - Selectivity Index (CC_{50}/EC_{50})

Figure 4: *In Vitro* Testing Results in MDCK cells

Conclusions

These studies conclude that NA subtype one (N1) has a higher NA activity comparative to NA subtype two (N2). The Duck virus is most resistant to oseltamivir in the NA-Star® assay and in cell culture studies. These data suggest there is correlation between NA activity and virus resistance to oseltamivir. This research will hopefully lead to better ways of identifying drug-resistant influenza virus strains.

Acknowledgments

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