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Full Length Research Paper

Mechanistic Insights into Sodium Periodate-Induced Inhibition of Influenza A Virus (H3N2) Attachment to Mammalian Cell Cultures

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Influenza epidemics cause numerous death and thousands of hospitalization each year. Because of the alarming emergence of resistant to anti-influenza drugs, there is a need to identify new anti- viral therapeutic agents. Viral tropism was stabilized in three mammalian celllines of different origin. The selected celllines are treated with sodium periodate at various concentrations to assess the rate of plaque reduction. Pretreated MDCK cells with sodium periodate at a concentration of 5 mM and 30% of plaque reduction was observed when compared to the untreated group. In A549 and Vero cells the plaque inhibition was found to be 11% and 22% respectively when compared with controls. The ability of influenza A virus (H3N2) binds to cells of canine, human and simian origin are reported here on the basis of cytopathic effect (CPE). H3N2 is more efficiently bound to cells of canine origin and the cytopathic effect was decreased with increasing the evolutionary complexity of the cell lines. The result suggested that dislodging of sialicacid receptors with sodium periodate were inhibiting the binding efficiency of human influenza A virus to mammalian cells.

Key words: Influenza A virus, sialicacid receptor protein, cytopathic effect, sodium periodate.

INTRODUCTION

Influenza A virus is a significant human pathogen that causes annual epidemics in the human population. The only two antigenic subtypes of influenza A virus circulate in human population namely H1N1 and H3N2 (Thompson et al., 2004). Human influenza A virus strain preferentially recognize sialicacid linked via an -2, 6 glycosidic linkage (2, 6) to the ultimate carbohydrate (Pekosz et al., 2009). The initial step in influenza virus infection is the firm attachment of a virus particle to the target cell surface which is accomplished through the interaction of a glyco-protein found on the viral surface (Hemagglutinin; HA), with cell-surface oligosaccharides containing sialicacids (Eisen et al., 1997). Many viruses recognize specific sugar residues, particularly sulfated or sialylated glycans, as the infection receptors. Avian influenza virus and human influenza virus use different sugar residues as reorganization sites, resulting in different host range of infections. Influenza viruses isolated and propagated solely in certain mammalian cell lines such as MDCK, Vero, LLC, MK2 and MRC-5 (Katz et al., 1990). It is generally believed that large glycoproteins better exposed on the cell surface are more likely to serve as receptor for the initial virus attachment, whereas subse-quent binding to gangliosides could bring the viral and cell membranes into closer proximity and thus, facilitate the membrane fusion which is a mandatory event for the entry of the viral genome into the cell (Matrosovich, 2006).

Influenza viruses are able to replicate in a variety of primary, diploid, and continuous cell cultures (Kilbourne, 1987). Although the susceptibility of most cell lines to influenza virus infection is low, human influenza viruses preferentially attaches to sialic acid (SA) with -2,6 galactose (-2,6 Gal) oligosaccharides (Rogers and Paulson, 1983; Carroll and Paulson, 1985) however, the distribution of these receptors on most mammalian cells has not been determined, and their influence on virus attachment and replication is still unclear (Govorkova et al., 1996). Nevertheless some strains of influenza virus A might grow extremely well in A549 cells. (Huang and

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Turchek, 2000). There are two strategies for blocking the attachment of a virus to the target cell; One is the blocking of the sugar-binding site of HA by peptides13 or Neu5Ac-containing derivatives. (Totani et al., 2003; Tsuchida, et al., 1998; Reuter et al., 1999; Guo, et al., 2002). (Sato et al., 2002) identified HA-binding peptides by using the phage-display system and showed inhibitory activity of the peptides for viral infections. Inhibitory activi-ties of Neu5Ac modified polymers (Totani et al., 2003) dendrimers, and lipids are also reported. These compounds bound to the Neu5Ac-binding site of HA and resulted in the inhibition of HA-Neu5Ac interaction (Tsuchida, et al., 1998; Reuter et al., 1999).

Sodium periodate has the ability to destroy carbo-hydrate moieties without altering the protein or lipid structures (Stevenson et al., 2004) Lymphocytes that have been transformed by sodium periodate, provide an excellent system for investigating alterations in surface structure. Since the cells have not been coated by foreign protein such as, lectins or antigens, sialicacid form a negatively charged sugar molecules usually found at the ends of oligosaccharides, attached to glycopoteins, glycolipids and proteoglycons. A number of viruses including enveloped and non-enveloped RNA and DNA viruses have been shown to use sialicacids as a compo-nent of their cellular receptor (Suzuki et al., 2000). Transduction of Madin-Darby bovine kidney (MDBK) cells pretreated with neuraminidase to remove cell surface sialicacid and with either sodium periodate to remove sialicacid conjugated carbohydrates (Li et al., 2009). The present study was therefore designed to define the interaction of influenza virus (H3N2) with specific population of cells *in vitro* and *in vivo.* Our result provides evidence for sialicacid as a component of influenza virus receptor; further more to find out viral tropism towards various cell lines of different origin.

MATERIALS AND METHODS

Virus

Human Influenza A (H3N2) were obtained from King Institute of Preventive Medicine and Research, Department of Virology, Chennai. It was propagated in MDCK cells as viral stocks.

Cell culture

Continuous MDCK, A549 and Vero cells were grown in minimal essential medium (MEM) contained 10% heat -inactivated fetal bovine serum (FBS) 100 Units/ml penicillin G and 100 g/ml streptomycin incubated at 37°C and 5% CO_2 for 72 h to get 90% confluency.

Test compound

Sodium periodate were purchased from Himedia chemicals. It was used in different dilutions to assess its cytotoxic concentration (CTC) . The compound prepared in ten different dilutions of 0.01 M to 0.1 M concentration for further assay.

Viral sensitivity assay

Confluent (90%) monolayer of MDCK, A549 and Vero cells were grown in six well plates. Ten fold serial dilution of H3N2 were used to infect each of the cell lines in six well plates. CPE was detected by fixation of cell monolayers with 4% formaldehyde in PBS and staining with 0.01% carbomyl fuscin solution.

Determination of effective minimal cytotoxic concentration of sodium periodate

Cytotoxicity of the compound against MDCK, A549 and Vero cells were evaluated in terms of CTC_{50} (50% cytotoxic concentration). MDCK, A549 and Vero cell cultures were exposed to the compound at ten different concentrations 0.01 to 0.1 M. Following 1 h of incubation at 37°C and washing with PBS, After 48 h incubation under the same conditions, the viability of the cells was measured by MTT method. Effective minimal cytotoxic concentration was determined by statistical analyses. The cell viability were assessed by using the formula:

$$\text{Cell viability} = \frac{\text{OD of the treated}}{\text{OD of control}} \times 100$$

Colorimetric MTT assay

Stock MTT (10x), was prepared by dissolving tetrazolium in PBS at pH 7.2 (Phosphate buffer saline) at a concentration of 5 mg/ml and filtered through 0.45 m of pore size.

The medium of the confluent cells was removed, then 100 l of 1x MTT was added to each well. Following incubation at 37°C with 5% CO_2 for 2 h, 100 l of acidic isopropanol was added and mixed to release the colour from the cells. Optical density was measured at 540 nm using ELISA reader (Stat Fax-200) to elevate live cells.

Plaque assay

Confluent MDCK, A549 and Vero cells were grown in six well plate and the cultures were treated subtoxic concentration of sodium periodate 0.01 M. After 1 h incubation 0.5 ml of viral suspension was added to both control and treated plates. Monolayer was inoculated with 0.5 ml of virus dilution, which was adsorbed for 1 h at 36°C. The inoculum was removed and the cells were washed twice with phosphate-buffered saline (pH 7.2) and were covered with 3 ml of an agar medium consisting 100 ml of 0.6% Agarose. After 2 days, a second agar overlay (1.0 ml) containing 1:1000 carbomyl fuscin was added to facilitate plaque counting. The same procedure was followed for A549 and Vero cells and plaque were counted for further analysis. Plaques are counted under microscope and the percentage of plaque reduction calculated by:

$$\%\text{ of Plaque reduction} = \frac{\text{Number of Plaque in treated}}{\text{Number of Plaque in control}} \times 100$$

RESULTS

H3N2 binds efficiently canine origin cells

Human influenza virus A (H3N2) was found to bind with all the selected mammalian cell lines. Among the three, MDCK cell was reported to be more susceptible to H3N2

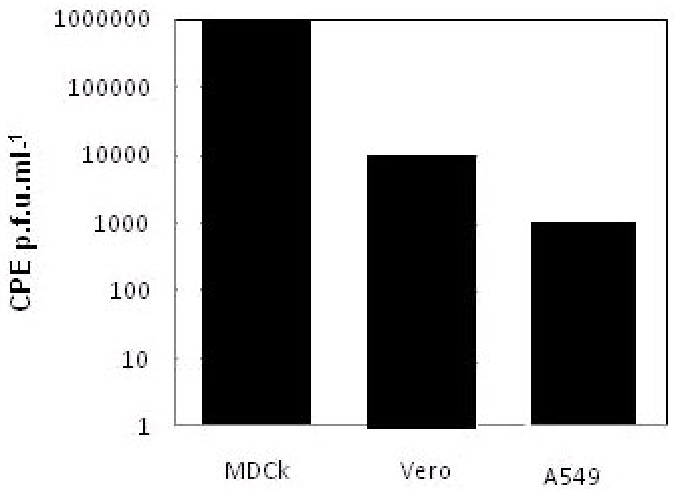

Figure 1. Ten fold serial dilutions of H3N2 were used to infect each of the cell lines in six well plates. CPE was detected by fixation of cell monolayers with 4% formaldehyde in PBS and staining with 0.1% carboyl fuscin. These experiments were repeated four times and one representative set of result is shown.

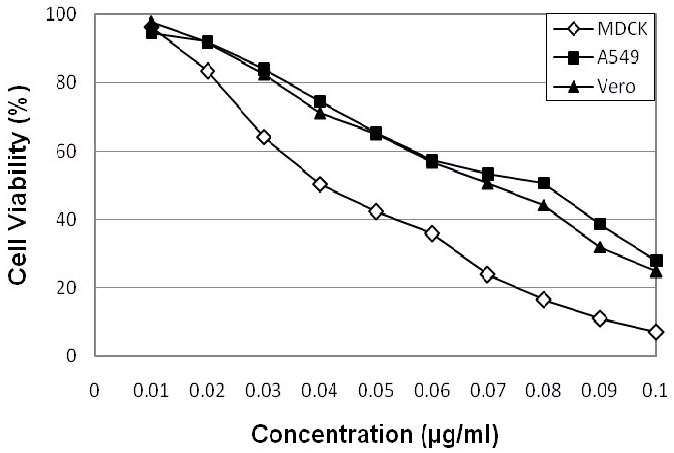

Figure 2. (CTC) effective minimal cytotoxic concentrations of sodium periodate. Optical Density at 540 nm (Y axis) of different dilutions of the compound (X axis) was measured by M.

strain and hence the cytopathic effect was found to be 10 lacks plaque forming units (pfu) per ml of cell culture medium. In the case of Vero and A549 cell lines, the cytopathic effect was expressed as 10,000 and 1000 pfu /ml (Figure 1). This part of result clearly demonstrated that the level of cytopathic effect due to H3N2 viral infection was 100 fold greater than the rest of selected two cells. Thus the cyotopathic effect was well pronounced in canine cells when compared to monkey and human cell lines.

MDCK cells contained numerous sialicacid species on their surface. A549 and Vero cells were observed to minimal availability of these silalicacid epitopes and their cell surface with carbohydrate moiety results negligible rate of cytopathic effect. The H3N2 influenza virus-infected Vero cells shown to have morphological changes in similar to those observed in MDCK cells. It was interesting both MDCK and A549 cells.

Data represented in Figure 2 clearly describing the subtoxic concentration of sodium periodate was determined as 0.1 µg/ml at which the cell viability was recorded. The cytotoxic effect was noticed at very close proximity for Vero and A549 cells, whereas, the MDCK cells observed to easily susceptible to sodium periodate than the rest of the two selected cells.

H3N2 binding / infection requires sialylated carbohydrate moieties

It has been shown that sodium periodate (NaIO4) destroyed carbohydrate moieties by oxidation of vicinal hydroxyl groups of sugars into dialdehydes at acidic pH without altering protein or lipid structures (Stevenson et al., 2004). To further examine the role of cell surface carbohydrate in H3N2 binding, MDCK, A549 and Vero cells were pretreated with sodium periodate which revealed the different levels of plaque reduction.

Pretreated MDCK cells with sodium periodate at a concentration of 0.005 M 30% inhibited compared to the untreated group. Whereas pretreated A549 and Vero cells with sodium periodate at the same concentration 11% in A549 and Vero cells 22% plaque inhibited com-pared to untreated controls(Figure 3) represents cytotoxic concentration 50 (CTC50) of sodium periodate in selected cell lines. Plaques formed by influenza A virus (H3N2) pre treated with subtoxic concentration of sodium periodate in selected cell lines (Figure. 4).

DISCUSSION AND CONCLUSION

Viruses should penetrate the host cells in order to cause infection. Like most of the enveloped viruses, the influenza virus use receptor binding and fusion as principal route of entry. The HA protein of the virus interact with the host cell sialicacid receptors and enters by receptor mediated endocytosis. Prevention of viral entry is an attractive anti-viral strategy as it can minimize the chance of virus evaluation and subsequent drug resistant ant strain development.

The earliest events in virus infection involve the interaction of virions with cell surface molecules. In this study we examined modulating the cell surface sialcacid receptor carbohydrate moieties with the sodium periodate were inhibiting the efficiency of virion-cell binding. Receptor specificity is an important mechanism governing the susceptibility of cells to virus infection. In the absence of the proper sialic acid receptors, influenza viruses may be unable to bind to the cell surface, thus eliminating the opportunity for productive infection. Although Vero cells

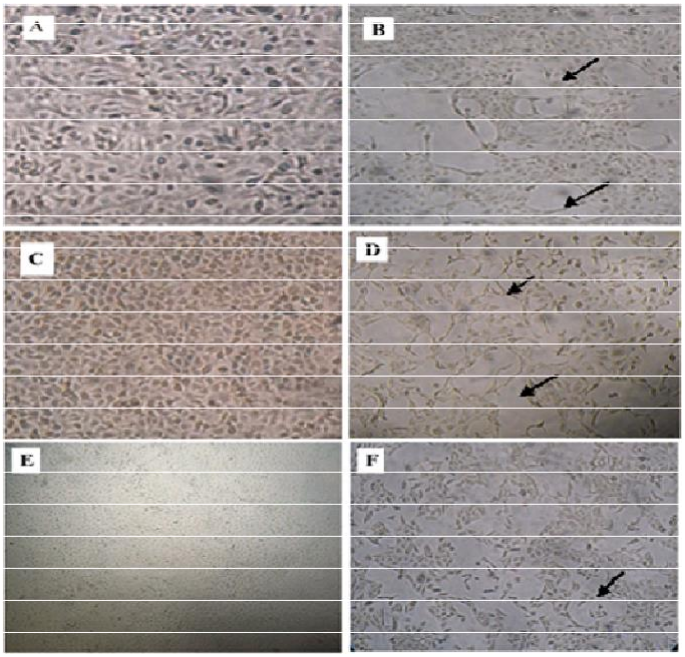

Figure 3. Cytotoxic concentration 50 (CTC $_{50}$) of Sodium periodate in selected cell lines; (A) Untreated control MDCK; (B) 0.04M treated Sodium periodate of MDCK with (CTC_{50}); (C) untreated A549; (D)0.08M treated A549 50% cytotoxicity; (E) Control Vero cells; (F) 0.07M exposed CTC_{50} of Vero cells.

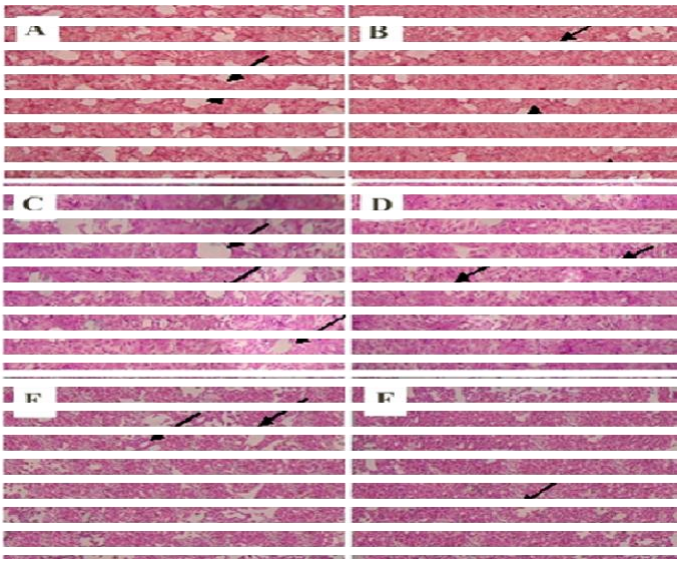

Figure 4. Plaques formed by influenza virus (H3N2) pretreated with subtoxic concentration of sodium periodate in MDCK, A549 and Vero cells respectively. (A) untreated control;(B) 0.01M treated MDCK; (C) untreated A549; (D) 0.01M treated A549; (E) untreated control; (F) 0.01M treated Vero cells.

bore a relatively low level of the NeuAc 2, 6 Gal linkages by comparison with MDCK cells, this relative abundance did not appear to affect their susceptibility to either influenza A or B viruses.

Govorkova, et al., (1996) finding raises the possibility that linkages other than NeuAc a2, 3 Gal and NeuAc a2, 6 Gal are involved in the attachment of influenza viruses to host cells.

These findings suggested that MDCK cells were very sensitive to sodium periodate it showed more cell death at low concentration of compound. An early study by (Pekosz et al., 2009) showed sialicacid play a vital role in binding of influenza A virus and interaction of HA with saccharides outside the terminal sialicacid. The alteration of the sialicacid and other saccharides can also affect the ability of HA to recognize sialicacid containing carbohy-drates (Russel et al., 2006).

Our results also shows that influenza virus binding is dramatically reduced upon pretreatment of selected cells with sodium periodate. Figure 1 demonstrate Neu5Ac 2-6 Gal and Neu5Ac 2- 3 Gal containing sialicacid species abundantly bound in MDCK cells (Gambaryan et al., 2005), Hence the H3N2 formed well cytopathic effect even low number of plaque forming units (pfu) of viral suspension. (Pekosz et al., 2009) also attempted to reduce the carbohydrates moieties on the cell surface receptor using O-glyconase and neuraminidase in combination, but the individual had no effect, we found that sodium periodate alone could reduce binding efficiency of H3N2 to its host.

Binging efficiency of H3N2 not only these but also depends upon the carbohydrate moieties present on the cell surface sialicacid receptor. Chu and Whittaker et al., (2004) stated influenza virus entry into susceptible cells appears to be dependent on sialicacid residues attached to N- linked carbohydrates. Therefore there may be a distinction between sialicacid residues that allow for binding of influenza residues that can mediate efficient entry of the virus, other carbohydrate residues besides the terminal sialicacid can contribute significant interact-tions with HA that can stabilize and facilitate viral binding (Nicholls et al., 2008).

This experiment shows that entry routes blocks the early viral binding to its receptor and viral fusion. We have proven experimentally that the efficient binding and fusion of H3N2 virus is required carbohydrate moieties present on the cell surface sialicacid receptor. Our study observed the differences in cell specific binding efficiency of H3N2 with selected mammalian cells, increasing evolutionary complexity of the cell lines and to resistant capacity increases against the particular viral strains.

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