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Interaction of Influenza A/H1N1pdm Virus with Human Neuronal and Ocular Cells

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Abstract

Background: Influenza virus is a highly prevalent respiratory virus responsible for annual epidemics as well as occasional pandemics. Extrarespiratory complications have been described, including neurological as well as ocular complications. This study examines the permissiveness of a panel of human neuronal and ocular cells to infection with the recent influenza A/H1N1pdm virus and cellular expression of α2,3- and α2,6-linked sialic acids.

Methods: Studied cells included the human neuroblastoma cell lines SH-SY5YplkOB1 and SK-N-SH, a glioblastoma patient isolate GBM847a, the human uveal melanoma cell lines OCM3, OMM1.5 and 92-1, and, as controls, Madin-Darby canine kidney (MDCK) and 16HBE human bronchial epithelial cells. Cells were infected with influenza A/H1N1pdm virus and their permissiveness was assessed via nucleoprotein (NP) staining analyzed by fluorescence microscopy. Additionally, cells were stained for the presence of α2,3- and α2,6-linked sialic acids and examined by fluorescence microscopy as well.

Results: All tested human cell types were permissive for influenza A/H1N1pdm infection. Furthermore, selected neuronal and ocular cells expressed α2,3- and/or α2,6-linked sialic acids.

Discussion: This study demonstrates the permissiveness of a panel of human neuronal and ocular cells for the recent influenza A/H1N1pdm virus and expression of α2,3- and α2,6-linked sialic acids. These data contribute to clinical as well as experimental evidence indicating that, apart from respiratory cells, influenza virus can infect other cells and, thus, may give rise to extrarespiratory complications.

Keywords: Influenza virus; Extrarespiratory; Neurological; Ocular; Neuroblastoma; Glioblastoma; Uveal melanoma; Neuroinvasion; CNS; Olfactory receptor neuron

Abbreviations: CNS: Central Nervous System, DAPI: 4',6-diamidino-2-phenylindole, DMEM: Dulbecco's Modified Eagle Medium; FBS: Fetal Bovine Serum; FCS: Fetal Calf Serum; IF: Immunofluorescence; IMDM: Iscove's Modified Dulbecco's Medium; LAS: Leica Application Suite; LM: Light Microscopy; MAL-I: Maackia Amurensis Lectin I; MAL-II: Maackia Amurensis Lectin II; MDCK: Madin-Darby Canine Kidney; MEM: Minimum Essential Medium; MOI: Multiplicity of Infection; NP: Nucleoprotein; ORN: Olfactory Receptor Neuron; PML: Progressive Multifocal Leukoencephalopathy; PVOD: Post-viral Olfactory Dysfunction; SNA: Sambucus Nigra Lectin; TCID50: 50% Tissue-culture Infectious Dose

Introduction

Influenza virus is one of the most prevalent respiratory viruses and responsible for annual epidemics as well as occasional pandemics with sometimes devastating outcomes. The virus is classically considered to mainly infect the upper, and occasionally lower, respiratory tract, clinically giving rise to upper respiratory symptoms and pneumonias. In addition to respiratory disease, extrarespiratory complications have been described, including neurological as well as ocular complications. Furthermore, associations between respiratory viral infections and the development of neuroinflammatory and neurodegenerative diseases have been postulated [1-6].

Whether or not extrarespiratory complications arise likely depends upon a delicate virus-host interplay involving distinct receptor interactions. In this respect, several viruses associated with central nervous system (CNS) and/or ocular complications, including adenoviruses, JC virus and influenza virus, interact with distinct sialic acids on host cell membranes [2,7-9]. Influenza viruses have, in terms of virus-host interactions, been classified into viruses that circulate in avian hosts and viruses circulating in other hosts including humans. In addition, viruses with low or high pathogenicity are distinguished [8]. Host cell binding and subsequent infection of cells by human influenza viruses are predominantly mediated by α2,6-linked sialic acids, whereas avian influenza viruses mostly interact with α2,3-linked sialic acids [8,9]. In this regard, the ability of avian H5N1 viruses to be transferred to -and among- human hosts, as well as issues involving receptor specificity and viral pathogenicity, have been the subject of recent investigation [10,11].

Neurological as well as ocular, often conjunctival, extrarespiratory complications have been described for both human and avian influenza viruses, although reported incidence rates for virus subtypes might vary [1,2,12-25]. The pathogenesis of these complications can, at least partly, be explained by receptor interactions. Upper respiratory tract epithelial cells mostly express α2,6-linked sialic acids, whereas cells lining the surfaces of the lower respiratory tract and eyes predominantly express α2,3-linked sialic acids, which might correlate with the observed clinical symptoms upon influenza virus infection *in vivo* [2,8,9]. The CNS potentially expresses both sialic acid subtypes as neurotropic viruses including JC virus, which can cause the demyelinating disorder progressive multifocal leukoencephalopathy (PML), have been shown to interact with α2,6- and α2,3-linked sialic acids [7]. Methods aimed

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at the mapping of sialic acids in several brain regions *in vivo* are being developed [7].

With respect to CNS entry routes, animal models have demonstrated that influenza viruses can infect the CNS via the olfactory system, likely following infection of olfactory receptor neurons (ORNs) within the olfactory neuroepithelium [6,26-33]. In humans, the syndrome of post-viral olfactory dysfunction (PVOD), consisting of olfactory loss and/or histological alterations in the olfactory epithelium, has been described following infections with respiratory viruses [34-36]. With respect to sialic-acid receptor expression patterns on ORNs, it has been shown that mouse ORNs can be stained with a pattern of lectins which recognize galactose, *N*-acetylgalactosamine and sialic acids, although subtypes and expression on human ORNs have not been determined [37].

This study examines the permissiveness of a panel of human neuronal and ocular cells for the recent influenza A/H1N1pdm virus, which has been associated with neurological as well as ocular complications [2,12,15-18], and analyzes sialic-acid expression patterns on these cells. Examined neuronal cells include the human neuroblastoma cell line SH-SY5YplkOB1, containing an empty plasmid, which was previously shown to be permissive for influenza A/H5N1 and seasonal H1N1 viruses and to express both α2,3- and α2,6-linked sialic acids [38]. The same study analyzed the permissiveness and sialic-acid expression of the human glioblastoma cell lines T98G and U87MG, whereas another study examined the permissiveness of the glioblastoma cell line GBM8401 to influenza B virus and, in the current study, we used the human glioblastoma patient isolate GBM847a [38,39]. The human neuroblastoma cell line, SK-N-SH, has been used to study the neuronal tropism of herpes viruses [40].

Examined ocular cells include the human uveal melanoma cell lines OCM3, OMM1.5 and 92-1. OCM3 and 92-1 cells were derived from primary tumors, and OMM1.5 cells from a metastasis [41]. Located within the eye, the uvea consists of the choroid, a cell layer neighboring the retina, capillary bodies and iris. The choroid provides nutrients and oxygen to the retina and uveal cells arise from the neuroepithelium or neural crest. In addition, these "non-neuronal" cells have been shown to have the capacity to differentiate to cells with a neuronal phenotype under appropriate culture conditions [42]. For these reasons, and particularly because of their close proximity to a cranial sensory nerve, i.e. the optic nerve, uveal cells may resemble ORNs.

With respect to the direct involvement of uveal melanoma cells in ocular complications, uveitis or uveal disorders have been associated with several viruses, including influenza virus [24]. Although a recent study showed H5N1 virus infection of ciliary process epithelial cells in ferrets, the permissiveness of human uveal cells to influenza virus infection and sialic-acid receptor expression patterns remain in question [2,43]. By analyzing the permissiveness to influenza A/H1N1pdm virus and sialic-acid receptor expression patterns of this panel of human neuronal and ocular cells *in vitro*, this study contributes to knowledge concerning neurological and ocular complications of influenza and possibly other viruses.

Materials and Methods

Cells and viruses

Human influenza A/CA/7/2009 (A/H1N1pdm) virus was propagated, and the TCID50 determined, in Madin-Darby canine kidney cells, as previously described [44,45].

The human neuroblastoma cell line SH-SY5YplkOB1, containing an empty plasmid, was a kind gift from Dr. J. Marie Hardwick (Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA). The human neuroblastoma cell line SK-N-SH was a kind gift from Dr. Jelena Levitskaya (Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA). The human glioblastoma patient isolate GBM847a was a kind gift from Dr. Alfredo Quinones-Hinojosa (Department of Neurosurgery, Johns Hopkins University, Baltimore, Maryland, USA). These cells were cultured using a mixture of Dulbecco's Modified Eagle Medium or Minimum Essential Medium (DMEM/MEM) (Life Technologies, Grand Island, New York, USA) and Ham's F12 medium (Life Technologies) supplemented with 10% fetal calf serum or fetal bovine serum (FCS/FBS), 100 U/mL penicillin and 0.1 mg/mL streptomycin (Life Technologies or PAA Labs, Pasching, Austria). Human uveal melanoma cell lines OCM3, OMM1.5 and 92-1 were a kind gift from Dr. Jelena Levitskaya and cultured using Iscove's Modified Dulbecco's Medium (IMDM, Life Technologies) supplemented with 10% FCS/FBS and 100 U/mL penicillin and 0.1 mg/mL streptomycin.

Madin-Darby canine kidney (MDCK) cells were cultured as described previously [44,45] or using Episerf medium (Life Technologies) supplemented with 25 mM Hepes (Life Technologies), 750 mg/L sodium bicarbonate (Life Technologies), 100 U/mL penicillin and 0.1 mg/mL streptomycin.

16HBE human bronchial epithelial cells were a kind gift from Dr. Herman Meurs (Department of Molecular Pharmacology, University of Groningen, The Netherlands). The cells were cultured using MEM, supplemented with 10% FBS, 2 mM L-glutamine, 1000 U/mL penicillin, 1 mg/mL streptomycin, 56 μg/mL gentamicin (Life Technologies) and 1.5 mg/mL fungizone (Life Technologies).

Cellular permissiveness

Cells were cultured on acid-washed cover slips and infected with influenza A/H1N1pdm virus at a multiplicity of infection (MOI) of 5 TCID50/cell for 1.5 hr at 37°C. Infection medium consisted of DMEM supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 4 μg/mL N-acetyl-trypsin. Following incubation at 37°C for 24 hr cells were fixed with 4% paraformaldehyde and stained for influenza nucleoprotein (NP), using mouse anti-NP [44,45] as a primary antibody and AlexaFluor 488 donkey anti-mouse (Life Technologies) as a secondary antibody, and embedded in mounting medium containing 4,6-diamidino-2-phenylindole (DAPI, Life Technologies or Vector Laboratories, Burlingame, California, USA). Controls consisted of similarly stained uninfected cells. Staining was analyzed by fluorescence microscopy using a Nikon Upright E800 microscope (Nikon, Chiyoda, Tokio, Japan) and Image-Pro Plus software (Media Cybernetics, Rockville, Maryland, USA).

Sialic-acid expression patterns

Cells cultured on acid-washed coverslips for 2 days at 37°C were fixed with 4% paraformaldehyde and stained with biotinylated Sambucus Nigra Lectin (SNA), recognizing α2,6-linked sialic acids or biotinylated Maackia Amurensis Lectin I or II (MAL-I or –II), recognizing α2,3-linked sialic acids [46] followed by AlexaFluor 488 or 647 conjugated to streptavidin (Vector Laboratories) and embedded in mounting medium containing DAPI. Controls consisted of cells stained with AlexaFluor-conjugated streptavidin alone. In addition, control human respiratory cells were incubated in 50 mU/mL neuraminidase from *C. perfringens* cleaving sialic acids on the cell surface in the order α2,3>α2,6>α2,8 (Sigma, St. Louis, Missouri, USA) for 1 hr at 37°C prior

to fixation and stained as above. Staining was analyzed by fluorescence microscopy, using a Nikon Upright E800 (see above) or Leica DM4000 B (Leica Microsystems GmbH, Wetzlar, Germany) and Image-Pro Plus or Leica Application Suite (LAS) software.

Results

Cellular permissiveness

To assess the permissiveness of human neuronal and ocular cells to influenza A/H1N1pdm virus infection, cells were cultured on acid-washed coverslips, infected at MOI 5 TCID50/mL, and incubated for 24 hr at 37°C. Cells were then stained for expression of influenza virus NP using a mouse anti-NP primary antibody and a donkey anti-mouse AlexaFluor 488 secondary antibody. To control for nonspecific staining, uninfected cells were stained according to a similar protocol. MDCK cells served as a positive control. Results were analyzed by fluorescence microscopy.

All tested human neuronal and ocular cells were permissive for influenza A/H1N1pdm infection (Figure 1). Positive cells included the SH-SY5Y and SK-N-SH human neuroblastoma, GBM847 glioblastoma patient isolate, and OCM3, OMM1.5 and 92-1 human uveal melanoma cells. MDCK cells stained positive as well (data not shown).

Sialic-acid expression patterns

To assess sialic-acid expression patterns on human neuronal and ocular cells, cells were cultured on acid-washed coverslips and stained for the presence of specific sialic-acid linkage subtypes. To assess the expression of α2,6-linked sialic acids, cells were stained with biotinylated SNA and to examine the presence of α2,3-linked sialic acids, cells were stained with MAL-I or MAL-II. Of the latter two lectins, MAL-I can be used to detect *N*-linked or core 2 *O*-linked glycans containing the trisaccharide Siaα2,3Galβ1,4GlcNAc and MAL-II to detect *O*-linked glycans containing the trisaccharide Siaα2,3Galβ1,3GalNAc [47]. To visualize biotinylated lectin binding, cells were stained with AlexaFluor 488 or 647 conjugated to streptavidin. Internal controls consisted of cells stained with AlexaFluor-conjugated streptavidin alone. As an additional control, human bronchial epithelial cells were treated with neuraminidase prior to staining. Staining results were analyzed by fluorescence microscopy.

Neuronal and ocular cells expressed distinct sialic-acid subtypes as indicated by positive lectin-staining results. Positive signals for α2,3-linked sialic-acid staining were observed for SH-SY5Y human neuroblastoma and OCM3 cells (Figure 2). Likewise, OMM1.5 and 92-1 human uveal melanoma cells stained positive for α2,3-linked sialic-acid (data not shown). The SK-N-SH human neuroblastoma cells and GBM847 patient isolate were not tested for α2,3-linked sialic-acid expression. In a similar fashion, positivity for α2,6-linked sialic-acid staining was demonstrated for SH-SY5Y and OCM3 cells (Figure 2). SK-N-SH human neuroblastoma, the GBM847 glioblastoma patient isolate, OMM1.5 and 92-1 human uveal melanoma cells also stained positive for α2,6-linked sialic-acid (data not shown). Human bronchial epithelial cells stained positive for α2,3-linked and, to a lesser extent, for α2,6-linked sialic acids. This staining was sensitive to treatment with neuraminidase from *C. perfringens*, underlining its specificity for sialic acid (Figure 3).

Discussion

Using a panel of different human neuronal, ocular and control cells, it was shown that these cells were permissive to influenza A/H1N1pdm virus infection and expressed α2,3- and/or α2,6-linked sialic acids. Although studied cell types are cell lines, they are of human origin and it will be interesting to see to which extent the current *in vitro* findings correspond to the human *in vivo* situation.

The observed overall permissiveness as well as sialic-acid receptor expression data of neuronal cells are in agreement with the results of a previous study in which different human neuronal cells, including SH-SY5Y as well as astrocytic cells, were shown to be permissive for highly pathogenic avian influenza A/H5N1 as well as low-pathogenic seasonal H1N1 viruses and to express both α2,3- and α2,6-linked sialic acids [38]. In the present study, SH-SY5Y and SK-N-SH neuroblastoma cells, as well as a GBM847 glioblastoma patient isolate, were permissive for the influenza AH1N1pdm virus and expressed α2,6- and/or α2,3-linked sialic acids.

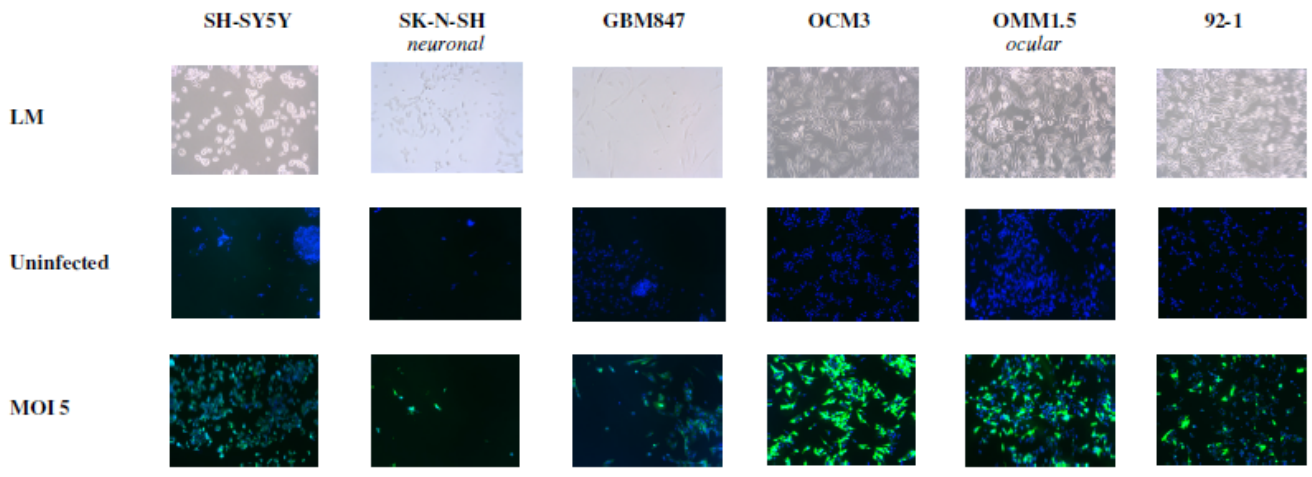

Figure 1: Permissiveness of human neuronal and ocular cells for influenza A/H1N1pdm virus. Cells were infected with influenza A/H1N1pdm virus at a MOI of 5 TCID50/mL and allowed to incubate for 24 hr. Following fixation, cells were stained with anti-NP primary and AlexaFluor-conjugated secondary antibodies, and embedded in mounting medium containing DAPI. Internal controls consisted of uninfected cells that were similarly stained. (LM: light microscopy, green: AlexaFluor 488; blue: DAPI, fluorescence microscope magnification: 100x).

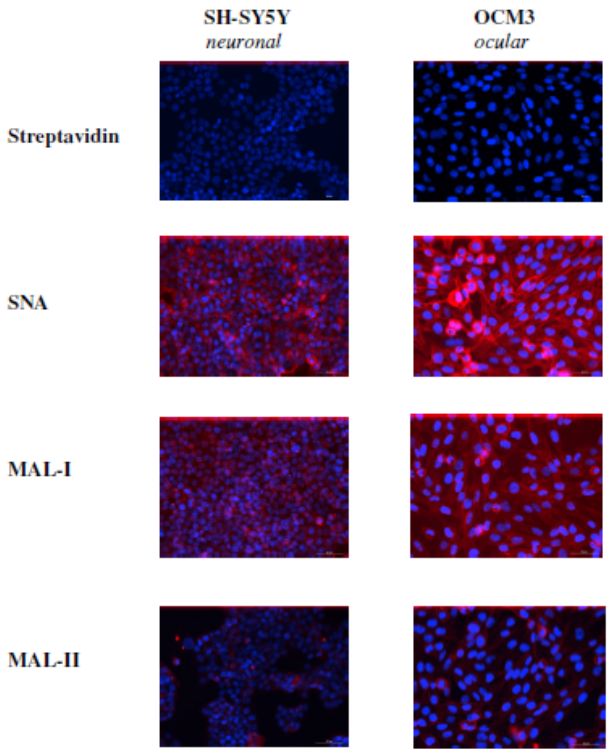

Figure 2: Sialic-acid expression on human neuronal and ocular cells. Following fixation, cells were stained with biotinylated lectins interacting with different sialic acid subtypes (SNA recognizing α2,6-linked sialic acids and MAL-I and -II recognizing α2,3-linked sialic acids) followed by staining with AlexaFluor-conjugated streptavidin, and embedded in mounting medium containing DAPI. Internal controls consisted of cells that were stained with streptavidin and DAPI alone. (red: AlexaFluor 647; blue: DAPI, fluorescence microscope magnification 200x).

With respect to ocular cells, previous studies have demonstrated the permissiveness of human corneal epithelial, trabecular meshwork, conjunctival epithelial and retinal pigment epithelial cells to, at least specific avian, influenza viruses [2]. In terms of receptor specificities, the cornea and conjunctiva predominantly express α2,3-linked sialic acids, whereas the nasolacrimal duct expresses both α2,3- and α2,6-linked sialic acids [2]. In the current study, uveal melanoma cells were permissive for influenza A/H1N1pdm virus and expressed α2,6- and α2,3-linked sialic acids. Susceptibility to infection may be clinically relevant because uveal complications have been described for several viral infections, including infections with influenza virus [24].

HBE cells expressed α2,3-linked and, to a lesser extent, α2,6-linked sialic acids. This binding pattern is consistent with the notion that human bronchi, bridging the upper and lower respiratory tract, might express both sialic acid subtypes [2,48].

With respect to the pathogenesis of extrarespiratory complications of influenza *in vivo*, viral as well as host factors likely play a role. In the case of neurological infections, both axonal and hematogenous spread of viruses to the CNS has been described. Axonal spread likely depends upon the ability of a virus to infect specific neuronal cell types, such as ORNs, and hematogenous spread on its capacity to elicit a viremia. Both CNS entry pathways have previously been demonstrated to be linked to distinct viral pathogenicity patterns [29]. With regard to ocular complications, similar virus-host interactions might play a role. In this respect, it has been demonstrated that influenza viruses, following ocular infection, can spread to other organ systems, including the respiratory tract [2].

The presence of influenza virus in the CNS in the context of apparent disseminated infections have been described for infection with avian H5N1 viruses, rendering hematogenous spread, at least for these viruses, a potential mechanism of viral dissemination to the CNS in humans [19-22]. Infection of ORNs and/or axonal spread along the olfactory nerve has been described for several viruses, including H5N1 and H1N1 influenza strains, in animal models of infection [6,26-33]. However, the exact sialic-acid receptor expression patterns and permissiveness of human ORNs remain to be determined. Although this study did not include ORNs, uveal melanoma cells are peripheral cells with a close embryological and anatomical linkage to the CNS and might resemble ORNs [42]. Using these cells, as well as several cells representing different CNS resident cell types, we have mimicked cell types ranging from the periphery to the CNS that might be encountered during axonal neuroinvasion, including invasion along the olfactory nerve. Receptor expression patterns on these cells might be of relevance to other viruses infecting the CNS and/or eyes, as adenoviruses and JC virus have been demonstrated to interact with sialic acids as well.

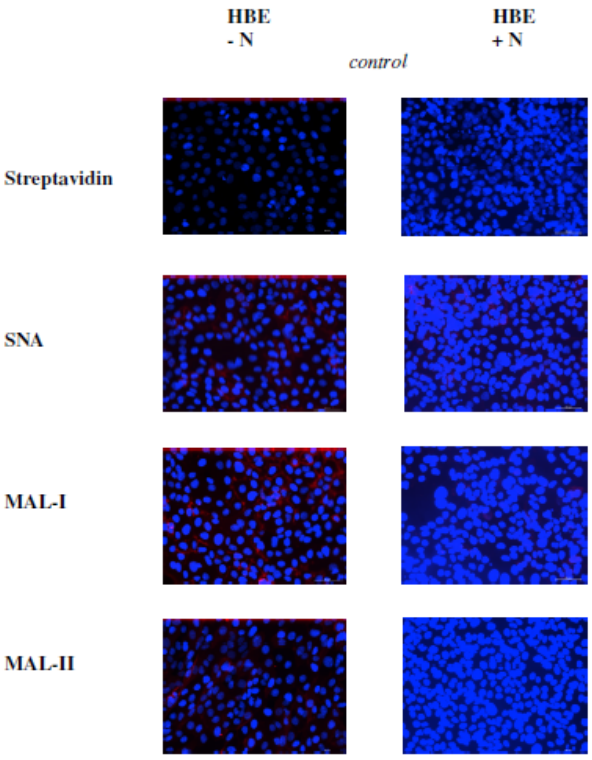

Figure 3: Sialic-acid expression on control human respiratory cells with and without neuraminidase treatment. Cells were treated with neuraminidase for 1 hr, fixed, and stained with biotinylated lectins interacting with different sialic acid subtypes (SNA recognizing α2,6-linked sialic acids and MAL-I and -II recognizing α2,3-linked sialic acids) followed by staining with AlexaFluor-conjugated streptavidin, and embedded in mounting medium containing DAPI. Controls consisted of cells that were not treated with neuraminidase and stained following a similar protocol. In both experimental settings, internal controls consisted of cells that were stained with streptavidin and DAPI alone. (N: neuraminidase; red: AlexaFluor 647; blue: DAPI, fluorescence microscope magnification 200x).

The present study contributes to clinical and experimental data demonstrating that, apart from infection of the respiratory epithelium, respiratory viruses can infect other cell types that are exposed to transmitted aerosols as well, with the possibility to cause extrarespiratory complications. It is plausible that respiratory viruses come into contact with ORNs or are deposited on ocular surfaces to extend infection, in conjunction with immunological and anatomical factors [2,26-28,49,50]. In this respect, it is noteworthy that several neurodegenerative/neuroinflammatory diseases start with dysfunction of one or more sensory systems, such as the olfactory or optic system [5,51,52]. Therefore, the pathogenesis of extrarespiratory complications and associated transmission routes deserve further investigation.

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