Receptor-binding variants of H3N2 influenza A viruses: characterization of their sialidase activity towards different substrates

Alessanda Abdo do Couto*
José Júnior França de Barros**
José Nelson dos Santos Silva Couceiro***

Abstract

Influenza virus sialidase develops an essential activity on cellular glycoproteins, then permitting the dissemination of the virus infections by preventing virus-virus self aggregation and virus-cell rebinding. Two purified variant samples of ***influenza*** A/Memphis/102/72 (H3N2) viruses, which are recognized for their receptor-binding activity to a-2,6 or a-2,3-sialyllactose structures, were analysed for their sialidase activity on different natural and artificial substrates. The M1/5 sample exhibited higher sialidase activity on fetuin (O.D.=0.226), MPN (O.D.=0.110) and human erythrocytes (10,240 HAU/ml), while the activity of the M1/5HS8 sample on these substrates was expressed by O.D.=0.129, O.D.=0.065 and 2,560 HAU/ml when using fetuin, MPN and human erythrocytes as substrates, respectively. However, the M1/5HS8 sample showed more significative sialidase activity on mucin when compared to the M1/5 sample: the enzyme activity of first sample was responsible for liberation of 3.5 nmol of free sialic acids while the last one produced 16.5 nmol of free sialic acids.

Keywords: Influenza virus. Receptor-binding variants. Neuraminidase (NA). Natural and artificial substrates.

INTRODUCTION

Influenza A virus is one of the most important re-emergent pathogens; it is responsible for flu outbreaks, epidemics and pandemics, conducing to significant morbity and mortality levels, and economic losses (LAMB; KRUG, 2001; POTTER, 1998). The RNA of these enveloped RNA viruses codifies for different glycoproteins, hemagglutinin (HA) and neuraminidase (NA, sialidase EC 3.12.1.18) both recognizing sialic acid structures. The 15 subtypes of HA spike mediates the binding of the virus particle to sialylglycoproteins and gangliosides of the host cell, while the 9 subtypes of NA act as receptor-destroying enzymes on sialic acids exposed on the same receptor carbohydrated structures (OKASAKI et al., 2000).

This virus sialidase is involved in the breakdown of the mucosal lining of the upper respiratory tract and cleavage of sialic acids from the respiratory tract mucus during the infection. It contributes to the release of the virion by the budding process and to prevent its entrapment in this substance (COUCEIRO et al., 1993;

* Bacharel em Microbiologia e Imunologia. Instituto de Microbiologia Prof. Paulo de Góes, CCS. UFRJ.
** Mestre em Ciências (Microbiologia). Instituto de Microbiologia Prof. Paulo de Góes, CCS. UFRJ.
*** Professor de Virologia. Doutor em Ciências (Microbiologia).
Instituto de Microbiologia Prof. Paulo de Góes, Laboratório de Virologia Molecular I. Departamento de Virologia. CCS, Bloco I. UFRJ.
21.941-590 - Rio de Janeiro RJ Brasil
Tel.: (21)2260-9311; Fax: (21)2560-8344
E-mail: jncouceiro@micro.ufrj.br

LIU et al., 1995; MURTI; WEBSTER et al., 1986). This tetrameric spike neuraminidase is a 200-240 kDa structure that exhibits a hydrophilic region with antigenic and catalytic sites and a hydrophobic one that is inserted into the virion lipid envelope (CRENNELL et al., 1993). From 1947, H3N2 influenza A virus samples have been selected by their affinity with a hemagglutinin receptor presenting specificity to NeuAc a-2,6Gal structures. The cleavage specificity of their sialidase has gradually drifted to hydrolyse both NeuAc a-2,6Gal and NeuAc a-2,3Gal structures. This is a selective advantage to the viruses, by more efficient destruction of cell receptors and releasing of newly formed virus particles (BAUM; PAULSON, 1991). This paper studies comparatively the sialidase activity of two A/Memphis/102/72 receptor-binding variants for their cleavage specificity towards different natural and artificial substrates.

MATERIALS AND METHODS

Purification of M1/5 and M1/5HS8 variants of ***influenza*** A/Memphis/102/72 (H3N2) viruses

The M1/5 variant and the M1/5HS8 variant, which were selected from M1/5 when it was replicated in Madin-Darby canine kidney (MDCK) cells maintained in presence of horse serum macroglobulin (ROGERS; PAULSON, 1983), were inoculated into 9-day-old embryonated chicken eggs and incubated for 48 hours at 34ºC. Allantoic fluids were harvested and clarified by centrifugation at 6,000 x ***g*** (Sorvall, GSA rotor) for 20 minutes, and concentrated at 80,000 x ***g*** (Beckman, SW40ti rotor) for 60 minutes. These samples were purified onto sucrose density gradient and finally twice washed at 100,000 x ***g*** (Beckman, SW40ti rotor) for 2 hours to remove sucrose residues. These purification steps were carried out according to Cabral and Couceiro (1994), and BARROS and others (2003).

To evaluate the efficiency of the purification process, the hemagglutinating activity of the virus samples at each purification step was observed at 4º C in triplicates, using 1% human erythrocytes (A group, Rh+) suspension in PBS with 0.2% BSA. Titers were read after 45 minutes and hemagglutination units (hemagglutinating unit/ml - HAU/ml) expressed as the reciprocal of the maximum dilution of virus that caused complete agglutination. Protein concentration of each purified sample was determined, either by the Bradford method (BRADFORD, 1976) or by absorption at 279 nm in a spectrophometer Beckman 1098, using bovine serum albumin as standard.

Characterization of the sialidase activity on natural substrates

Activity on mucin as substrate

Sialidase activity on mucin was assayed by a colorimetric method, submitting both variant samples at different times of incubation (2 and 4 hours), pH (5.2, 5.4, 5.6 and 5.8) and calcium concentrations (2 and 4 mM), using fetuin as substrate. In this assay, a 25 ml–volume of each sample was mixed with 50 ml-volume of mucin (10 mM) as substrate in phosphate buffer at different conditions of pH and calcium concentration and submitted at different incubation times as already described. After incubation at 37ºC, the assay to evaluate the liberated Neu5Ac residues was carried out according to the periodic acid/TBA method described by Warren (1959) with modifications by Aymard-Henry and others, (1973) as described by Barros and others (2003), plotting the value of optical density at 549 nm (OD_{549}) versus concentration of standard Neu5Ac solutions (0.5 to 10 mg in 50 ìl samples).

Substrate specificity on a-2,3-sialyllactose, a-2,6-sialyllactose and fetuin as substrates

The activity of the sialidase activity of both variants on fetuin and sialyl-lactose isomers at 1.5 mM was analysed by periodic acid/TBA method, using those best conditions of calcium concentration, pH ambient and time incubation as already described above (BAR-

ROS et al., 2003), with spectrophotometrical analysis of the sialic acid residues liberated in the supernatant at 405 nm (Pharmacia spectrophotometer, Novaspec II model).

Substrate specificity on cells

Sialidase activity of viral NA on human erythrocytes (type A Rh+) was determined by microassay according to Lothan and others (1975). The purified virus samples were serially diluted in 25 ml-volumes of PBS, pH 6.4 containing 6 mM $CaCl_2$ and 0.2% BSA, to avoid an interference on cell integrity, and incubated with 25 ml-volumes of 1% erythrocytes at 37°C for 3 hours. The treated red cells with ***Vibrio cholerae*** (50 U/ml) were then resuspended, mixed with 25 ml-volume PNA solution (4 HAU) and incubated at 4°C for 30 minutes. The sialidase titer was expressed as the reciprocal of the maximum dilution of enzyme where complete hemagglutination (HAU/ml) by the lectin was observed (BARROS et al., 2003).

Substrate specificity on artificial substrate

The enzyme hydrolysis activity was also analysed on an artificial substrate, 2-(3-methoxyphenyl)-N-acetyl-D-neuraminic acid (MPN). In this methodology, a 60 ml-volume of each diluted purified sample (1:20) in phosphate buffer at ideal conditions of pH, calcium concentration and time incubation was mixed with an equal volume of 0.3 mM MPN in the same phosphate buffer. After incubation at 37°C, a 60-ml volume of 3 mg/ml of Fast Black K salt in phosphate buffer was added. The mixture was incubated for 2 hours in ice bath and the resulting chromogen was solubilized in 1.5 ml-volume of 0.5 mM NaOH and determined spectrophotometrically at 580 nm. The 3-methoxiphenol liberated (LEE; SEONG, 1998) and the enzyme activity was measured by OD readings taken from the part of the slope where they vary linearly with the enzyme concentration.

RESULTS AND DISCUSSION

The purified samples exhibited hemagglutinating titers from 640 HAU/ml (M1/5 variant) to 2,560 HAU/ml (M1/5HS8 variant); their protein content was adjusted to 0.5 mg/ml for analysis of sialidase activity (data not shown).

The enzymatic behavior of this viral sialidase on mucin was studied during 2 and 4 hours of incubation, at different pH ambients (5.2, 5.4, 5.6, and 5.8) and two different calcium concentrations (2 and 4 mM). The ideal conditions for analysis of sialidase activity of both H3N2 variants were obtained at pH 5.4, in presence of 4 mM of calcium after 2 hours of incubation at 37°C (data not shown). The pH 5.4 and 2 mM of calcium should be due to permit the best conformational structure and calcium concentration respectively for the NA enzyme activity; in these conditions the M1/5 and M1/5HS8 variant samples exhibited activities on mucin that were expressed by 3.5 and 16.5 free sialic acids per nmol respectively (FIGURE 1). These results are very probably

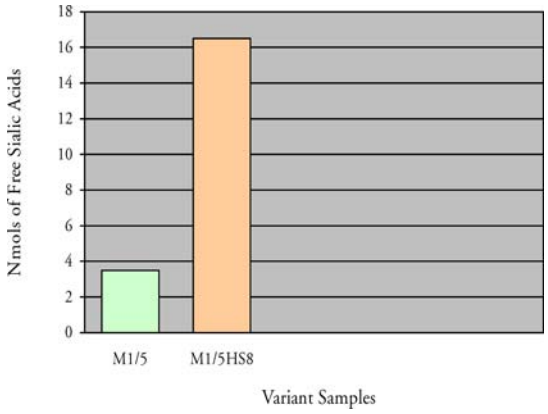

Figure 1 - Sialidase activity of M1/5 and M1/5HS8 samples of influenza A/Memphis/102/72 viruses for mucin as substrate. The study was developed in triplicates, analysing nmols of free sialic acids liberated after 37°C incubation for 4 hours at pH 5.4.

Note: The study was developed in triplicates, analysing nmols of free sialic acids liberated after 37°C incubation for 4 hours at pH 5.4.

due to recognition of abundant SA-a2,3Gal-b1,4GlNac sequences present in human bronquial mucin (LAMBLIN et al., 1984; BREG et al., 1987; COUCEIRO; PAULSON; BAUM, 1993).

The analysis of the sialidase activity of the variants on substrates as á-2,6-sialyllactose and á-2,3-sialyllactose showed an evident specificity of cleavage on both structures, however showing a minor activity on á-2,3-sialyllactose ones. The enzyme activity of the M1/5 sample was responsible for optical densities as 0.432 and 0.175, when it was expressed on á-2,3-sialyllactose and á-2,6-sialyllactose sequences, respectively. The M1/5HS8 also exhiited similar optical densities, expressing its enzyme activity on á-2,3-sialyllactose (0.393) and á-2,6-sialyllactose (0.173) structures (TABLE 1).

The sialidase activity on fetuin exhibited a differential behavior, the enzyme activity of the M1/5 sample was due to 0.226 as optical density, while the activity of the M1/5HS8 sample was expressed by 0.129 as optical density (TABLE 1). Fetuin is a tetrasaccharide structure composed by ***N***-acetylneuraminyl-(2,3)-b-D-galactopyranosyl-(1,3)***N***-acetylneuraminyl (2, 6)-a-D-***N***–acetylgalactosaminyl-(1,3) which is bounded to serine (SPIRO; BHOYROO, 1974).

As already observed on the fetuin, significantly different optical densities were observed when the M1/5 (0.110) and M1/5HS8 (0.065) samples respectively were analysed for their silalidase activity on MPN (2-(3-methoxyphenyl)-N-acetyl-D-neuraminic acid), which are also exhibited in Table 1.

The M1/5 and M1/5HS8 variants of ***influenza*** A/Memphis/102/72 virus differ from each other in their receptor-binding specificity due to a single amino acid change at residue 226 in the hemagglutinin; the M1/5 variant sample preferentially recognizes the SA-a-2,6Gal sequences while the M1/5HS8 variant recognizes the SA-a-2,3Gal ones (BAUM; PAULSON, 1991; COUCEIRO; PAULSON; BAUM, 1993; BARROS et al., 2003). The receptor or sialidase specificities of the hemagglutinin and neuraminidase activities to SA-a-2,6Gal and SA-a-2,3Gal sequences and the sequence present in higher amount on the cell surface explain the ability of those virus samples to bind or replicate into mammalian or avian cells that expose higher number of SA-a-2,6Gal or SA-a-2,3Gal surface structures respectively (ROGERS; PAULSON, 1983; PINTO; CABRAL; COUCEIRO, 1994). By 1972, ***influenza*** virus N2 NA had developed similar specificities for both NeuAc-a-2,6Gal and NeuAc-a-2,3Gal structures, acquiring a same linkage of sialic acid that is preferentially recognized by their HA to prevent self aggregation of virus and to facilitate the efficient release of virus progeny from cells (KILBOURNE; JOHANSSON; GRAJOWER,1990; BAUM; PAULSON, 1991).

The sialidase activity on human erythrocytes was demonstrated here by hemagglutination of desialylated erythrocytes with PNA. The enzyme titers expressed by hemagglutination titers on previously desialylated erythrocytes were 10,240 and 2,560 HAU/ml for the M1/5 and M1/5HS8 variants respectively (FIGURE 2). The titers obtained with the M1/5 and M1/5HS8 variants

Table 1 - Sialidase activity of M1/5 and M1/5HS8 samples of influenza A/Memphis/102/72 viruses for substrates as a-2,3-sialyllactose, a-2,6- sialyllactose, fetuin, and MPN

Variant samples	Activity on substrates[1]			
	α-2,3-Gal siallyllactose	α-2,6-Gal sialyllactose	Fetuin	MPN
M1/5	0,432	0.175	0.226	0.110
M1/5HS8	0.393	0.173	0.129	0.065

[1]The analysis of sialidase activity of the M1/5 and M1/5HS8 variant samples was developed in triplicates, by spectrophotometrical analysis (O.D.) of their products of activity on α-2,3 structures, α-2,6 structures, fetuin (549 nm), and on MPN (580 nm) as substrates.

revealed that they are able to hydrolyse both NeuAc a-2,3Gal and NeuAc a-2,6Gal structures, however the results could be due to conclude for a differential activity.

The M1/5 sample of influenza A/ Memphis/102/72 exhibited sialidase activity four times higher than its M1/5HS8 variant when using native human erythrocytes as target cells. This observation can be explained for its higher affinity to a-2,6-sialyllactose structures, which are mainly expressed on human erythrocytes.The sample M1/5 exhibited higher sialidase activity on fetuin, MPN and human erythrocytes, while the M1/5HS8 sample showed more significative sialidase activity on mucin when compared to the M1/5 sample.

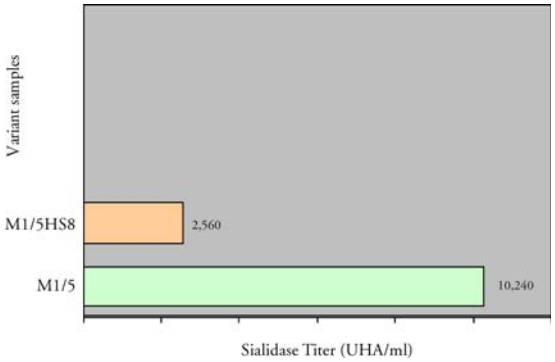

Figure 2 - Sialidase activity of M1/5 and M1/5HS8 samples of influenza A/Memphis/102/72 viruses on human erythrocytes. The study was developed in triplicates, analysing the number of hemagglutinatin units (HAU/ml) observed after expression of the sialidase activity on these cells for 3 hours at 37°C.

Note: The study was developed in triplicates, analysing the number of hemagglutinatin units HAU/ml) observed after expression of the sialidase activity on these cells for 3 hours at 37°C.

Variantes de receptor de virus influenza H3N2: caracterização de sua atividade sialidásica sobre diferentes substratos

Resumo

A atividade sialidásica do vírus influenza tem uma atividade essencial sobre glicoproteínas celulares, permitindo a disseminação de infecções virais por prevenir a auto-agregação entre partículas virais e a re-ligação vírus-célula. Duas amostras variantes purificadas de vírus influenza A/Memphis/102/72 (H3N2), reconhecidas por sua atividade de ligação a receptores apresentando estruturas como a2,6 ou a2,3-sialilactose, foram analisadas por sua atividade sialidásica sobre diferentes substratos naturais e artificiais. A amostra M1/5 mostrou maior atividade sialidásica sobre fetuína (D.O.=0,226), MPN (D.O.=0,110) e eritrócitos humanos (10.240 unidades hemaglutinantes/ml), enquanto a atividade da amostra M1/5HS8 foi expressa por D.O.=0,129, D.O.=0,065 e 2.560 unidades hemaglutinantes/ml quando usados, respectivamente, fetuína, MPN e eritrócitos humanos como substratos. Contudo a amostra M1/5HS8 exibiu uma atividade sialidásica mais significativa sobre mucina quando comparada à amostra M1/5; a atividade enzimática da primeira amostra foi responsável pela liberação de 3,5 nmol de ácidos siálicos livres, enquanto a última produziu 16,5 nmol de ácidos siálicos livres.

Palavras-chave: Vírus influenza***. Variantes de receptor. Neuraminidase (NA). Substratos naturais e artificiais.***

REFERENCES

AYMARD-HENRY, M. et al. Influenzavirus neuraminidase and neuraminidase-inhibition test procedures. ***Bull. World. Health.Organ.***, Geneva, v.48, n.2, p.199-202, 1973.

BARROS, J. J. F. et al. Characterization of sialidase from an influenza A (H3N2) virus strain: kinetic parameters and substrate specificity. ***Intervirology***, Basel, v.46, p.199-206, 2003.

BAUM, L. G.; PAULSON, J. C. The N2 neuraminidase of human influenza virus has acquired a substrate specificity complementary to the hemagglutinin receptor specificity. ***Virology***, New York, v.180, p.10-15, 1991.

BRADFORD, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. ***Anal. Biochem***., Orlando, v.72, p.248-154, 1976.

BREG, J. et al. Structure of sialyloligosaccharides isolated from bronchial mucus glycoproteins of patients suffering cystic fibrosis. ***Eur. J. Biochem***., Berlin, v.168, p.57-68, 1987.

COUCEIRO, J. N. S. S.; PAULSON, J. C.; BAUM, L. G. Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity. ***Virus Res***., Amsterdam, v.29, p.155-165, 1993.

CRENNELL, S. J. et al. Crystal structure of a bacterial sialidase from ***Salmonella typhimurium*** LT2) shows the same fold as an influenza virus neuraminidase. ***Proc. Natl. Acad. Sci. USA***, Washington, DC, v.90, p.9852-9856, 1993.

KILBOURNE, E. D.; JOHANSSON, B. E.; GRAJOWER, B. Independent and disparate evolution in nature of influenza A virus hemagglutinin and neuraminidase glycoproteins. ***Proc. Natl. Acad. Sci. USA***, Washington, DC, v.87, p.786-790, 1990.

LAMB, R. A.; KRUG, R. M. Orthomyxoviridae: the viruses and their replication. In: KNIPE, D. M. et al. (Ed.). ***Fields' virology***. 4th. ed. Philadelphia: Lippincott Williams & Wilkins, 2001. p.1487-1531.

LAMBLIN, G. et al. Primary structure determination of five sialylated oligosaccharides derived from bronchial mucus glycoproteins of patients suffering from cystic fibrosis. J. ***Biol. Chem***., Baltimore, v.259, p.9051-9058, 1984.

LEE, K.; SEONG, B. The position 4 nucleotide at the 3'end of the influenza virus neuraminidase vRNA is involved in temporal regulation of transcription and replication of neuraminidase RNAs and affects the repertoire of influenza virus surface antigens. ***J. Gen. Virol***., London, v.79, p.1923-1934, 1998.

LIU, C. et al. Influenza type A virus neuraminidase does not play a role in viral entry, replication, assembly or budding. ***J. Virol***., Washinton, DC, v.69, p.1099-1106, 1995.

LOTHAN, R. et al. The purification, composition, and specificity of the anti-T lectin from peanut (***Arachis hypogaea***). ***J. Biol. Chem***., Baltimore, v.250, p.8518-8523, 1975.

MURTI, R. G.; WEBSTER, R. G. Distribution of hemagglutinin and neuraminidase on influenza virions as revealed by immunoelectron microscopy. ***Virology***, New York, v.149, p.36-43, 1986.

OKASAKI, K. et al. Precursor genes of future pandemic influenza perpetuated in ducks nesting in Siberia. ***Arch. Virol***., Wien, v.145, p.885-893, 2000.

PINTO, A. M. V.; CABRAL, M. C.; COUCEIRO, J. N. S. S. Hemagglutinating and sialidaseactivities of subpopulations of influenza A viruses. ***Braz. J. Med. Biol. Res.,*** São Paulo, v.27, p.1141-1147, 1994.

POTTER, C. W. Chronicle of influenza pandemics. In: NICHOLSON, K. G.; WEBSTER, R. G.; HAY, A. J. (Ed.). ***Textbook of influenza***. London: Blackwell Science, 1998. p.1-18.

ROGERS, G. N.; PAULSON, J. C. Receptor determinants of human influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. ***Virology***, New York, v.127, p.361-373, 1983.

SPIRO, R. G.; BHOYROO, V. D. Structure of the ***O***-gycosidically linked carbohydrate units of fetuin. ***J. Biol. Chem***., Baltimore, v.249, p.5704-5717, 1974.

WARREN, L. The thiobarbituric acid assay of sialic acids. ***J. Biol. Chem***., Baltimore, v.234, p.1971-1975, 1959.

Acknowledgements

Grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Universitária José Bonifácio (FUJB/UFRJ), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Financiadora de Estudos e Projetos (FINEP) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).