Glycosylation influencing on fusion activity of HA and HEF structures of influenza viruses

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Abstract
Since the most ancient times, influenza viruses have caused lethal respiratory diseases. Their replication process occurs at the epithelial cells of the respiratory tract, where the surface-anchored glycoproteins hemagglutinin (HA) and hemagglutinin-esterase-fusion protein (HEF) of influenza A and C viruses respectively are responsible for the fusion process. The adsorption, sialidase and esterase activities developed by these same structures are inborn, while the fusion process is dependent of previous glycosylation and protein cleavage. Indeed, the glycosylation is strictly related to antigenicity and stability of fusion proteins. This work was designed to analyse the influence of the de-glycosylation for the development of fusion activity, using influenza A and C viruses as study models. The de-glycosylation provoked significative reduction in the fusogenic activity, inducing a reduction equal to 51.0%, 87.5%, 95.5% and 79.3% for A/Memphis/102/72, A/FM/1/47, C/Taylor/1233/47 and C/Paris/1/67 respectively. However, this activity was improved at certain pH values, 10.1% (pH 5.8), 59.4% (pH 5.8), 32.5% (pH 5.8) and 80.7% (pH 5.4) for the 95.7% at pH 5.2 for A/Memphis/102/72, A/FM/1/47, C/Taylor/1233/47 and C/Paris/1/67 of influenza viruses respectively. The fusogenic activity of certain samples was also improved at some pH values. These results permit to conclude that the level of glycosylation is closely related to the protein stability and the de-glycosylation process causes a significative influence on the fusion biological activity.

Keywords: Influenza viruses. Hemagglutinin (HA). Hemagglutinin-esterase-fusion protein (HEF). Fusion activity. Glycosylation.

INTRODUCTION

In the family Orthomyxoviridae, the four genera of influenza viruses are identified by antigenic characteristics of their nucleoprotein (NP) and protein matrix (M). These virus particles present segmented -RNA, capsid of helicoidal simmetry and one or two glycoprotein structures inserted in their lipoprotein envelope (LAMB; KRUG, 2001; HORIMOTO; KAWAOKA, 2001). For influenza A viruses, HA spikes are related to adsorption and fusion processes, while NA exhibit sialidase activity (HUGHES et al., 2001). Otherwise, the influenza C virus particles present only HEF spikes exhibiting adsorption, fusion and esterase activities (HERRLER; KLENK, 1991).

During the virus replication cycle, the adsorption process is followed by endocytosis;

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the virus particle is taken into an endosome where previous cleaved HA0 (HA1 and HA2) or HEF0 (HEF1 and HEF2) are responsible for fusion between virus envelope and endosome membrane, where a fusogenic peptide is exposed at acid pH (5.0-6.0 pH range), finally releasing the virus nucleocapsid into the cytosol. The nucleocapsid is then transported into the nucleus where transcription and RNA replication take place, which are followed by translation, assembly and budding processes (HERRLER; KLENK, 1991; LAMB; KRUG, 2001).

Each monomer of trimeric HA molecule presents a long stem and a globular head responsible for fusion and adsorption-fusion activities respectively. Each HA molecule contains from three to nine N-linked oligosaccharide, depending of the virus sample. The number and localization of oligosaccharide residues on the head vary for the different influenza A subtypes, while oligosaccharide residues are scarce and extremely conservative at the stem. HA presents three types of N-linked oligosaccharides bound to an asparagine residue: one type that is manose-riched, another one composed by fucose, galactose and glucosamine residues, and the hybrid type. The glycosylation also stabilizes the HA structures during the intracellular transport, trimerization, assembly and maturation (HOSOYA et al., 1992; LAMB; KRUG, 2001; SKEHEL; WILEY, 2000; SCHULZE, 1997; OHUCHI, M. et al., 1997; OHUCHI, M.; OHUCHI, R.; MATSUMOTO, 1999; ROBERTS; GARTEN; KLENK, 1993).

Each monomer of the HEF trimeric molecule presents receptor and esterase sites localized in loops above the stem, which exhibits a partially exposed fusogenic peptide. Six N-linked oligosaccharides on the HEF1 portion were identified as responsible for adsorption and esterase activities while only one was identified in the fusogenic HEF2 portion. The HEF glycosylation is mainly related to protein stabilization, protecting it from proteolytic degradation and improving the presentation of its antigenic epitopes (FORMANOWSKI et al., 1990; HERRLER; KLENK, 1991).

This work intends to evaluate the influence of the glycosylation on fusion biological activity developed by surface structures of influenza viruses, using influenza A and C viruses as models.

MATERIALS AND METHODS

Preparation of virus samples

Influenza A/Memphis/102/72 (H3N2), A/FM/1/47 (H1N1)] and C [C/Paris/1/67 and C/Taylor/1233/47] virus samples were propagated in 7 to 9-day old chicken embryonated eggs. After 48 and 72 h of incubation at 34ºC, the allantoic fluids were collected, clarified (6,000 x g for 30 min. at 4-6°C) and 50 times concentrated at 80,000 x g for 1h at 4-6ºC. The concentrated preparations were purified in a 20-60% sucrose gradient, at 100,000 x g for 2h at 4-6ºC and finally dialysed against phosphate buffer saline (PBS). The influenzavirus samples were standardized for their protein content at 0.05 mg/ml in a sodium citrate buffer 0.1 M, then finally exhibiting 538.9 (A/Memphis/102/72), 168 (A/FM/1/47), 8.6 (C/Taylor/1233/47) and 27.8 (C/Paris/1/67) HAU (hemagglutinating units) per 25 ml, the sample titer (HAU/25ml) corresponding to the inverse of the higher dilution where total human erythrocytes (A group, Rh+) agglutination was observed (COUCEIRO, J. N.; BAUM, 1994). The samples were stored at -20ºC until using (MINUSE; QUILLIGAN Jr.; FRANCIS Jr., 1954; MURPHY; KIES, 1960; BARRETT; INGLIS, 1987; COUCEIRO, J. N.; COUCEIRO, E. S. S.; MACHADO, 1987; COUCEIRO, J. N.; MACHADO; COUCEIRO, E. S. S., 1990).

The samples were treated by endoglycosidase H (10 mIU) for 18 h at 37°C, in the controls (non treated), the amount of enzyme was substituted for buffer. The endoglycosidase H or endo-b-N-acetylglucosaminidase H (enzyme classification number: 3.2.1.96) cleaves between two residues of N-acetylglucosamine in the diacetylchitobiose core of manose-riched or hybrid oligosaccharides. Complex oligosaccharides containing galactose, sialic acid and fucose are completely resistant to this enzyme

(ROBBINS et al., 1977; COLLINS; KNIGHT, 1978; TRIMBLE; MALEY, 1984).

Fusion activity analysis

The analysis of the fusion process on cellular membranes was developed through hemolysis of human erythrocytes, with spectrophotometric measure of the hemoglobin liberated in the process. At first, a suspension of human erythrocytes at 1% was added to those different virus samples in triplicates, with incubation in ice-bath for 30 minutes. Then, acetate buffers (sodium chloride 0.154 M and sodium acetate 50 mM) at different pH values (5.0/5.2/5.4/5.6/5.8/6.0) were added, with incubation to 37°C for 20 minutes and centrifugation at 4,500 rpm for 10 min. Fusion activity was revealed by the spectrophotometrical analysis of the hemoglobin liberated in the supernatant, using a Pharmacia spectrophotometer (Novaspec II model) at 405 nm. Each assay was developed in triplicates for twice, considering the arithmetical average of the data (Ab) as the final result of each experiment. The fusion percentuals of each virus sample at each pH were finally calculated, using those values of absorbance (arithmetical average) related to virus fusion activity (A: virus + erythrocytes + buffer), residual absorbance (B: erythrocytes + buffer) and maximal absorbance (C: erytrocytes + buffer with 0.1% Triton X-100) as observed in the following equation: [A – B / C – B] x 100 (CUBEL; COUCEIRO, J. N.; CABRAL, 1992).

RESULTS AND DISCUSSION

The fusogenic activity of the A/Memphis/102/72 (H3N2), A/FM/1/47 (H1N1), C/Taylor/1233/47 and C/Paris/1/67 samples at 5.0-6.0 pH range, after treatment with endoglycosidase H as their controls, is observed in Figures 1 and 2.

The influenza A and C samples showed many profiles of fusogenic activity, depending on that pH ambient used in test. Expressive reductions can be observed: 51.0% for the A/Memphis/102/72 sample at pH 6.0 (Figure 1A), 87.5% for the A/FM/1/47 sample at pH 5.4 (Figure 1B), 95.5% for the C/Taylor/1233/47 sample at pH 5.2 (Figure 2A), and 79.2% for the sample C/Paris/1/67 at pH 5.2 (Figure 2B). However, fusogenic activity was increased for those A/Memphis/102/72 (10.15% at pH 5.8 – Figure 1A), A/FM/1/47 (52.3 % and 59.4% at pH 5.8 and pH 6.0 respectively – Figure 1B), C/Taylor/1233/47 (32.5% at pH 5.8 – Figure 2A) and C/Paris/1/67 (80.7% at pH 5.4 – Figure 2B) samples.

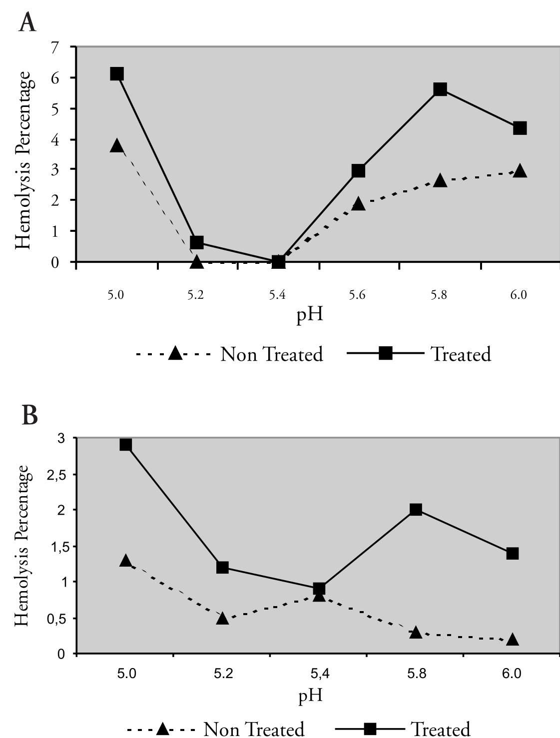

Figure 1 - Effect of de-glycosylation treatment on the fusogenic activity presented by A/Memphis 102/72 (A) and A/FM/1/47 (B) samples of influenza viruses

Note: The analysis of endoglycosidase-treated and non reated virus samples was developed in triplicates,by spectrophotometrical (405 nm) measurement of the hemoglobin liberated in the supernatant.

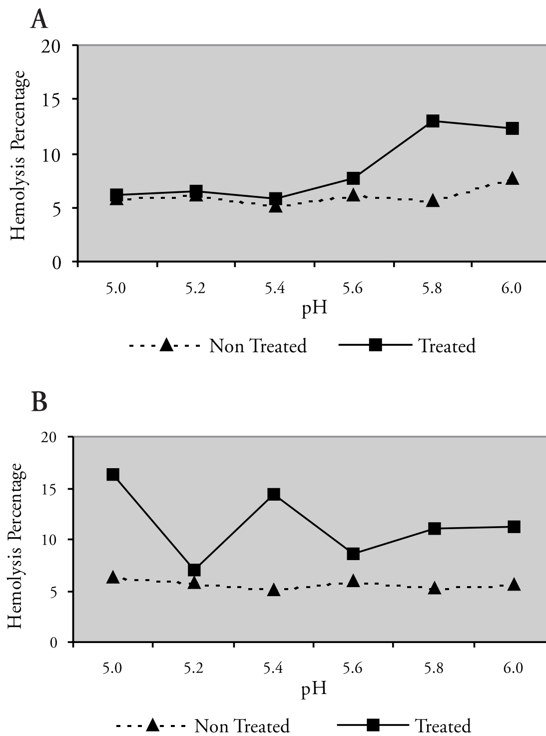

Figure 2 - Effect of de-glycosylation treatment on the fusogenic activity presented by C/Taylor/1233/47 (A), and C/Paris/1/67 (B) samples of influenza viruses

Note: The analysis of endoglycosidase-treated and non treated virus samples was developed in triplicates, by spectrophotometrical (405 nm) measurement of the hemoglobin liberated in the supernatant.

Reduction of the fusogenic activity can be explained by endoglycosidase cleavage; the cleavage of oligosaccharides localized next to the viral receptor site can be responsible for a higher affinity between HA and its receptor (sialic acid), not allowing that conformational change of HA trimmer heads, which is essential for fusion between viral and cellular membranes (OHUCHI, M.; OHUCHI, R.; MATSUMOTO, 1999). Higher hemolysis percentages were exhibited for samples treated with endoglycosidase, indicating that this enzyme has acted on oligosaccharides residues close to the fusogenic peptide at the stem (OHUCHI, R. et al., 1997), probably making it a more efficient tool for the fusion process.

It is important to notice after endoglycosidase treatment that, although virus samples like A/Memphis/102/72, A/FM/1/47 and C/Taylor/1233/47 at that standard protein concentration (0.05 mg/ml) exhibited nule hemagglutinating titer and low or nule enzymatic activity (sialidase or esterase), such preparations still showed fusogenic activity. This behavior reveals that the minimal ratio among virus particles and cells to provoke hemagglutination is larger than the virus amount to provoke fusion, revealing non dependence between the different activities of HA e HEF functions, absence of one activity permitting the expression of another one.

So, the glycosylation is able to reduce the adsorption, fusion and sialidase actvities, however some sample exhibited reduction of the fusogenic biological activity at certain pH values. These results permit to conclude that the glycosylation level shows influence on the conformational stability of proteins, since the deglycosylation commonly reduced the biological activities. In the same way, the importance of the glycosylation level was already demonstrated for the surface proteins of measles and respiratory syncytial viruses (OGURA et al., 1991; COLLINS; MOTTET, 1992). It was observed that Respirovirus and Rubulavirus genera present N-linked glycosylation sites in both the F1 and F2 subunits of their fusion protein (F), while members of the Morbillivirus genus exhibits N-linked glycans only in the F2 subunit (MICHALSKI et al., 2000).

Genetic expression and molecular techniques have shown to be more efficient as well as have shown to be specific tools to observe the real importance of the glycosylation for the fusion activity performance. In future, these methodologies can be useful to obtain more detailed knowledge on this subject.

Glicosilação influenciando a atividade de fusão de estruturas HA e HEF de vírus influenza

Resumo
Desde os tempos mais remotos, os vírus influenza têm sido o agente causal de doenças respiratórias letais. O seu processo de replicação ocorre nas células epiteliais do trato respiratório, causando uma síndrome respiratória aguda, na qual as glicoproteínas hemaglutinina (HA) e hemaglutinina-esterase-fusão (HEF), ancoradas na superfície dos vírus influenza A e C, respectivamente, são responsáveis pelos processos de fusão durante o ciclo de replicação viral. Ao contrário dos processos de adsorção, sialidase e esterase, atividades inatas desempenhadas por estas mesmas estruturas, a fusão depende de uma glicosilação e clivagem protéica prévias, para que o vírus se torne infeccioso. A glicosilação das proteínas de superfície está associada principalmente com a antigenicidade e com a estabilidade das proteínas de fusão. O presente trabalho analisa a influência da de-glicosilação sobre a atividade de fusão, usando vírus influenza A e C como modelos de estudo. A de-glicosilação parcial provocou significativa redução da atividade fusogênica, induzindo uma redução de 51,0%, 87,5%, 95,5% e 79,3% em relação às amostras de vírus influenza A/Memphis/102/72, A/FM/1/47, C/Taylor/1233/47 e C/Paris/1/67, respectivamente, acarretando também eventuais aumentos desta atividade biológica para certas amostras: 10,15% (pH 5,8), 59,47% (pH 5,8), 32,55% (pH 5,8) e 80,7% (pH 5,4) com relação às amostras de vírus influenza A/Memphis/102/72, A/FM/1/47, C/Taylor/1233/47 e A/Paris/1/67, respectivamente. Os resultados permitem concluir que o nível de glicosilação mostra-se estreitamente relacionado com a estabilidade das proteínas, causando o processo de-glicosilação uma influência significativa sobre a atividade fusogênica viral.

Palavras-chave: *Vírus influenza. Hemaglutinina (HA). Hemaglutinina-esterase-fusão (HEF). Atividade de fusão. Glicosilação.*

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