GENETIC STABILITY OF THE ATTACHMENT GLYCOPROTEIN OF HUMAN RESPIRATORY SYNCYTIAL VIRUSES DURING SERIAL PASSAGES IN CELL CULTURES

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Summary. – Thirteen isolates of human respiratory syncytial viruses (HRSV) of groups A and B were isolated in HEp-2 cells from nasopharyngeal aspirates (NPA) from the children with acute respiratory infections. Three isolates of HRSV of group A were propagated in HEp-2 cells in 20 serial passages. Nucleotide sequences of the products obtained by RT-PCR from the glycoprotein (G) hypervariable region of the original virus isolates in NPA and those after one or several passages were compared. All the isolates analyzed showed no changes during passaging in HEp-2 cells.

Key words: human respiratory syncytial viruses; genetic stability; attachment glycoprotein

Introduction

Human respiratory syncytial viruses (HRSV), members of the species *Human respiratory syncytial virus (*the genus *Pneumovirus*, the subfamily *Paramyxovirinae*, the family *Paramyxoviridae*) (van Regenmortel *et al.,* 2000) are the leading cause of severe infectious diseases of respiratory tract of infants and young children worldwide, producing yearly large epidemics (Collins *et al*., 2001). HRSV contain a non-segmented negative-sense single-stranded RNA that encodes at least ten proteins.

Panels of monoclonal antibodies (MAbs) have been used to subdivide HRSV into two antigenic groups A and B (Anderson *et al.*, 1985; Mufson *et al.*, 1985), which correspond to genetically distinct viruses (Cristina *et al.*, 1990). High genetic diversity of the attachment G gene occurs between and within the HRSV groups A and B (reviewed by Melero *et al.*, 1997). Thus, studies on molecular epidemiology of HRSV have focused on the G protein showing several evolutionary lineages among isolates of both groups (Cane *et al.*, 1991; García *et al.*, 1994; Peret *et al.*, 1998; Matinez *et al.*, 1999; Venter *et al.*, 2001; Frabasile *et al.*, 2003).

Accumulation of sequence changes at certain positions of the G protein reflect immunological selection of new variants (García *et al.*, 1994; Cane and Pringle, 1995; a review by Melero *et al.*, 1997). Thus HRSV can overcome the pressure of antibodies raised after natural infection by generating variants with changes in the epitopes of the G protein, and also by selection of HRSV escape mutants resistant to anti-G protein MAbs (García Barreno *et al.*, 1990; Rueda *et al.*, 1991, 1994) or polyclonal antibodies (Sullender and Edwards, 1999). The changes identified in the HRSV G protein of the variants by immune selection could be single amino acid substitutions or more drastic changes including frame-shift mutations or premature stop codons (reviewed by Melero *et al.*, 1997).

For several RNA viruses, increased genetic and antigenic variations have been reported in the absence of immune selection, as was observed in immunocompromised hosts during chronic or persistent infections established by a rotavirus (Hundley *et al.*, 1987), coxsackie virus (O´Neil

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Abbreviations: FMDV = Foot-and-mouth disease virus; G = glycoprotein; HRSV = human respiratory syncytial viruses; IF = immunofluorescence; MAb(s) = monoclonal antibody(ies); NPA = nasopharyngeal aspirates; PIV3 = Parainfluenza virus 3

et al., 1988), Foot-and-mouth disease virus (FMDV) (Gebauer *et al.*, 1988), and Measles virus (Catteneo *et al.*, 1986). Also, there has been reported a selection of antigenic variants of FMDV (Diez *et al.*, 1989) during serial passaging in cell cultures, and a FMDV rescued from BHK-21 cell lines persistently infected with FMDV (Diez *et al.*, 1990).

Here, in order to evaluate the effects of passaging HRSV in the absence of immune pressure, we compared the sequences of amplified viral G RNA obtained from the virus isolates in NPA with those after 1 and 20 passages of the virus isolates in cell cultures.

Materials and Methods

Virus isolation. Thirteen RSV immunofluorescence (IF)-positive NPA specimens from children under five years of age admitted at the Public Children´s Hospital "Pereira Rossell" in Montevideo, Uruguay with acute lower respiratory tract infections, were inoculated onto monolayers of HEp-2 cells. Three isolates were then further passaged 20 times in HEp-2 cells.

Total RNA was extracted directly from clinical samples and from inoculated HEp-2 cells with TRIZOL® according to the manufacturer´s instructions. Briefly, 1 ml of TRIZOL® was added to each cell pellet or 400 µl of a NPA, followed by vortexing. After 5 mins of incubation 200 µl of chloroform was added, the mixtures were vortexed for 3 mins, and the lysates were centrifuged in a microcentrifuge at 14,000 rpm for 15 mins. The supernatants were collected and precipitated with 0.5 ml of isopropanol per supernatant for 10 mins and centrifuged. at 14,000 rpm for 10 mins The obtained pellets from infected HEp-2 cells were dried and resuspended in 50 µl of sterile distilled water per pellet, while those from NPA were resuspended in 8 µl of sterile distilled water containing 5 U/µl Rnasin. The RNAs were stored at -70°C until used in RT-PCR.

RT-PCR and nucleotide sequencing of the G gene of HRSV. The HRSV G protein gene was amplified from the extracted total RNA. To produce the first strand of cDNA the RT reaction was run at 37°C for 1 hr in the first strand buffer (250 mmol/l Tris-HCl pH 8.3, 375 mmol/l KCl, 15 mmol/l $MgCl_2$, 50 mmol/l DTT) containing 20 µg of total RNA, 10 mmol/l dNTPs, 0.1 mol/l DTT, 300 ng of the antisense primer LG3(-) (5´-GGCCCGGGAAGCTTTTTTT TTTTTTTT-3´) with an *Ava*I site (underlined) for cloning purposes, and 200 U of Moloney murine leukemia virus reverse transcriptase (Promega). The amplification of the entire G gene cDNA proceeded in 100 µl of the PCR buffer (200 mmol/l Tris-HCl pH 8.4 and 500 mmol/l KCl) containing 5 U of Taq DNA polymerase (Gibco- BRL), 2 µl of the first strand cDNA, 300 ng of the antisense LG3(-) as well as the sense primer LG5(+) (5´-GGAT CCCGGGGCAAATGCAAACATGTCC-3´) with an *Ava*I site and the first 20 nucleotides of the G gene from the reference Long strain (Johnsom *et al*., 1987), and 50 mmol/l $MgCl_2$.

In order to amplify the C-terminal third of the G gene, a seminested PCR was performed with a PCR product using the sense primer GA480(+) 5´-ACAAACCACCAAACAAACCC-3´ or GB496(+) 5´-GATGATTACCATTTTGAAGTGTTCA-3´, for samples from HRSV groups A and B, respectively. In the same way, using the first amplified product with primers LG3 and LG5, but to amplify the N-terminal part of the G gene, a heminested PCR was carried out with the primer GA573(-) 5´-GCAGATGGCC CAGCAAGTTGG-3´. The reactions were run in a Thermolyne Amplitron® I under these conditions: one cycle at 99°C/5 mins, followed by 35 cycles at 94ºC/90 secs (denaturation), 54°C/2 mins (annealing), 72°C/1 min (extension), and 72°C/10 mins (final extension).

The PCR products were purified using either the Wizard ® PCR Preps DNA Purification System (Promega) or the CONCERT Gel extraction System (Gibco-BRL).

The PCR products were sequenced using ^{32}P-labeled dideoxynucleotides and the cycling sequencing as recommended by the manufacturer (Amersham) or they were directly sequenced in an ABI PRISM™ 377 Sequencer using the Applied Biosystems Fluorescent Dye Terminator Kit with 5 pmoles of primers.

Data analysis: alignment and p-distance. Nucleotide sequences of HRSV isolates of groups A and B were aligned separately with Clustal X 1.8 (Higgins and Sharp, 1988; Thompson *et al*., 1997), applying the multiple alignment option. P-distances were calculated by MEGA 2.1 (Kumar *et al*., 2001).

Nucleotide sequence accession number. The sequences analyzed in this study have been submitted to the GenBank with the following accession numbers: AF516117 to AF516120, AY571776 to AY571781, AY488794, AY488795, AY488798.

Results and Discussion

Thirteen HRSV isolates, ten of them isolated in 1997, belonging to the group A and three of the group B isolated in 1999, were analyzed after one passage in HEp-2 cells. Three isolates of the group A (Mon/3/97, Mon/5/97, Mon/6/97) were further passaged in HEp-2 cells in total 20 times (Table 1). Total RNA was extracted directly from NPA, after one and 20 passages, reverse transcribed and the G gene was amplified by PCR.

G was selected to evaluate the HRSV variability in the absence of immune pressure, because the latter seems to display the highest genetic diversity between and within the HRSV groups A and B (reviewed by Melero *et al.,* 1997).

The hypervariable C-terminal third of the G protein gene from all the isolates was sequenced (Fig. 1). Also the N-terminal region constituting the entire G gene sequence of the three isolates passaged 20 times was sequenced.

Principal differences between the sequences analyzed corresponded to (i) point mutations located mostly at the variable region of the ectodomain of the G protein, and (ii) changes in the stop codon position.

The deduced amino acid sequences of the group B isolates indicated that all of them shared the G protein length of 295 amino acids, while among the group A isolates the G length of 297 or 298 amino acids was detected. In all cases of both groups the stop codon was an UAG triplet (Fig. 2).

Table 1. Comparison of the sequences of viruses present in original clinical samples with those in the viruses passaged in HEp-2 cells

Antigenic group	Strain[a]	Sample analyzed[b]	Sequenced region[c]	Result of comparison
A	Mon/3, 5, 6/97	NPA isolate, passage 20	a/b	Identical
	Mon/1, 2, 4, 7-10/97	NPA and isolate	b	Identical
B	Mon/1, 2, 5/99	NPA and isolate	c	Identical

Mon = Montevideo; NPA = nasopharyngeal aspirate (original clinical sample).
[a]Strains are designated by the place of isolation, number and year of isolation.
[b]Sample analyzed refers to NPA isolate.
Passsage 20: the virus passaged 20 times in HEp-2 cells.
[c]See Fig. 1.

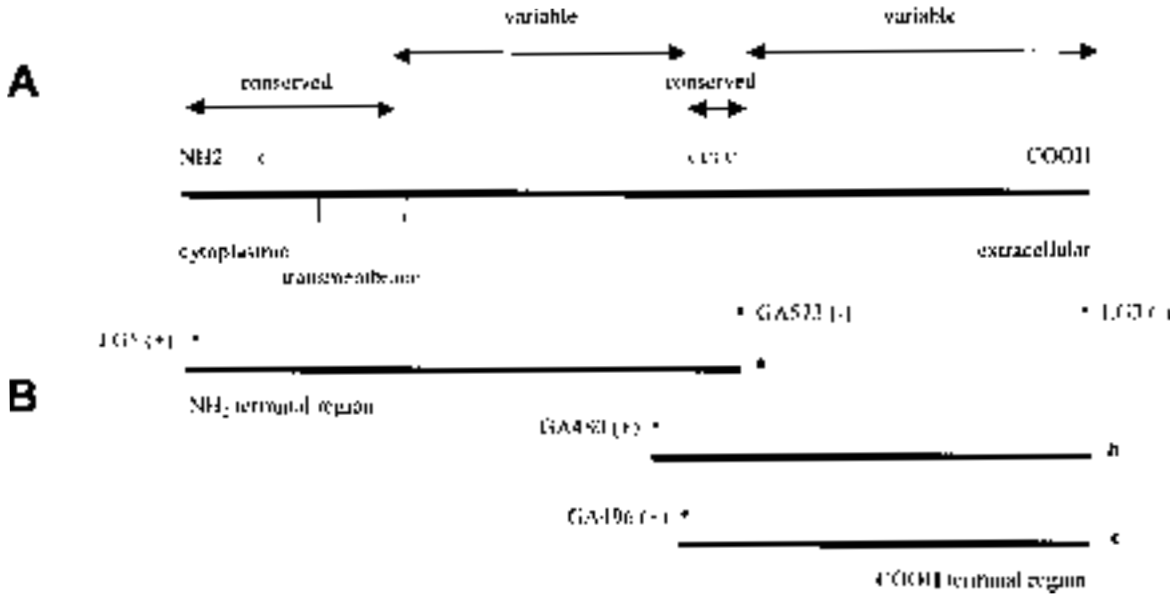

Fig. 1

A linear representation of the attachment glycoprotein G and the sequenced genome fragments

A. Antigenic domains of the HRSV G. Two variable regions of the ectodomain flank a highly conserved central domain of four cysteins (c).
B. The sequenced genome fragments of isolates of groups A (b) and B (c), passaged once in HEp-2 cells, and those in their NPA ancestors. The amino-terminal region sequenced only (i) in the three isolates passaged 20 times in HEp-2 cells, (ii) in their first passage in HEp-2 cells, and (iii) in their NPA ancestors.

The genetic p-distances between individual sequences within each group ranged from 0.00 to 0.074 and from 0.013 to 0.031 for groups A and B, respectively (Table 2). Some values of p-distance suggest that isolates from the same epidemic period were not necessarily most closely related, as it has been reported earlier (Cane and Pringle, 1995; Garcia *et al.*, 1994; Melero *et al.*, 1997). E.g., the isolates Mon/1/97 and Mon/2/97 with the p-distance of 0.061 clustered in the phylogenetic analysis separately, belonging to different genotypes (Frabasile *et al.*, 2003).

Each isolate passaged once in Hep-2 cells was compared with its ancestor in NPA (Table 1). The results showed that the sequence of the G gene of each isolate did not change as a result of one passage *in vitro*. Also, no changes were detected after 20 serial passages in Hep-2 cells, indicating high genetic stability of the attachment G of HRSV in the absence of immune pressure. These results are in agreement with those previously obtained by Cane *et al.* (1994), who have concluded that the observed diversity of the G gene of HRSV isolates was not due to in vitro propagation of the viruses.

Amino acid position								
HRSV isolates	292	293	294	295	296	297	298	299
Group A								
HRSVLong	CCA	CCC	AAC	ACA	ACA	CGC	CAG	**UAG**
Mon/1/97	CCA	UCC	AAC	AUA	ACA	AAC	CAG	**UAG**
Mon/2/97	CCA	UCC	AAC	ACA	ACA	AAC	**UAG**	**UAG**
Mon/3/97	CCA	UCC	AAC	AUA	ACA	AAC	CAG	**UAG**
Mon/4/97	CCA	UCC	AAC	ACA	ACA	AAC	**UAG**	**UAG**
Mon/5/97	CCA	UCC	AAC	AUA	ACA	AAC	CAG	**UAG**
Mon/6/97	CCA	UCC	AAC	AUA	ACA	GAC	CAG	**UAG**
Mon/7/97	CCA	UCC	AAC	AUA	ACA	GAC	CAG	**UAG**
Mon/8/97	CCA	UCC	AAC	AUA	ACA	GAC	CAG	**UAG**
Mon/9/97	CCA	UCC	AAC	ACA	ACA	AAC	**UAG**	**UAG**
Mon/10/97	CCA	UCC	AAC	AUA	ACA	GAC	CAG	**UAG**
Group B								
CH18537	AAU	**UAA**	AAA	ACC	**UAG**			
Mo/1/99	ACC	CAA	AAA	CUC	**UAG**			
Mon/2/99	ACC	CAA	AAA	CUC	**UAG**			
Mon/5/99	ACC	CAA	AAA	CUC	**UAG**			

Fig. 2

Termination codon usage by Uruguay HRSV isolates

Partial sequence of the C-terminal end of the G mRNA of reference strains from group A and B, Long and CH18537, respectively (Johnson *et al*., 1988), were included for comparison. Stop codons are indicated in bold.

Table 2. Genetic p-distance between individual sequences within groups A and B of HRSV isolates

Group A	Mon/3/97	Mon/1/97	Mon/5/97	Mon/10/97	Mon/8/897	Mon/7/97	Mon/6/97	Mon/4/97	Mon/9/97
Mon/3/97									
Mon/1/97	0.005								
Mon/5/97	0.000	0.005							
Mon/10/97	0.038	0.043	0.038						
Mon/8/97	0.025	0.029	0.025	0.014					
Mon/7/97	0.034	0.038	0.034	0.023	0.014				
Mon/6/97	0.025	0.029	0.025	0.018	0.005	0.014			
Mon/4/97	0.068	0.072	0.068	0.068	0.061	0.070	0.061		
Mon/9/97	0.070	0.074	0.070	0.070	0.063	0.072	0.063	0.002	
Mon/2/97	0.061	0.065	0.061	0.059	0.054	0.063	0.054	0.016	0.018
Group B	Mon/5/99	Mon/2/99	Mon/1/99						
Mon/5/99									
Mon/2/99	0.013								
Mon/1/99	0.031	0.030							

To determine the genetic variability of the viruses, each isolate was compared with its NPA ancestor but not compared with each other. Moreover, the genetic changes identified in each isolate were point mutations located mostly at the variable region of the ectodomain of the G protein.

The sequences of the G gene of each isolate obtained directly from clinical samples and after one passage in HEp-2 cells, showed no changes (Table 1). These results are in agreement with those previously obtained by Cane *et al.* (1994), who have observed by restriction analysis that five HRSV isolates both from original specimens and after propagation in tissue cultures showed no genetic changes. Also, several reports on influenza A and B viruses using direct sequencing of the hemagglutinin gene in original clinical samples and those isolated from passages in mammalian cells revealed a sequence identity (Katz *et al.*, 1990; Katz and Webster, 1992; Robertson *et al.*, 1990, 1991). However, it should be mentioned that when HRSV or influenza viruses are propagated in different cell lines, the viruses isolated from each cell line show significant differences. Thus, infection of different human epithelial cell lines with HRSV revealed changes in both electrophoretic mobility and antigenicity of the G protein (García-Beato *et al.*, 1996). Influenza viruses from infected individuals grown in embryonated chicken eggs and in mammalian Madin-Darby canine kidney cell culture result in viruses, which differ antigenically and structurally (Schild *et al.*, 1983; Meyer *et al.*, 1993).

In our study, no changes were detected in the entire G gene after 20 serial passages in cell culture (Table 1), indicating high genetic stability of the attachment G of HRSV in the absence of immune pressure.

These results are in sharp contrast with those found for other viruses where the effect of serial passage resulted in divergence from original virus. Thus, a selection of antigenic variants (i) of FMDV upon serial passaging in cell culture (Diez *et al.*, 1989), (ii) of a virus rescued from BHK-21 cell lines persistently infected with FMDV (Diez *et al.*, 1990), and (iii) of HSV-1 during persistent infection in cell culture (Hampar *et al.*, 1967) have been reported. However, our results are in agreement with previous observations made by Cane et al. (1994) during a study of variability of HRSV isolates, proving that the observed diversity of the G gene of HRSV isolates was not due to *in vitro* culturing of the virus. In these experiments, a clinical sample underwent one passage in HEp-2 cells, three passages in MRC-5 cells, eleven passages in MRC-5 cells, and 11 passages in BSC-1 cells. Only one non-coding change at nucleotide 30 (G to A) in the virus that was passaged 11 times in BSC-1 cells was observed. The attempts to amplify the G gene from the original clinical sample were unsuccessful.

A high stability in the F protein of Parainfluenza 3 virus (PIV3), besides HRSV another member of the Paramyxoviridae family, has been reported (Zambón *et al.*, 1998). Thus, during a molecular epidemiological study of a PIV3 outbreak in a Bone Marrow Transplant Unit, four immunosuppressed patients shed virus persistently for 1–4 months without change in the respective sequence. In this case the PIV 3 strain variation was analyzed by nucleotide sequence of a portion of the F gene, which has a high degree of sequence diversity relative to the other parts of the PIV3 genome, including the hemagglutinin-neuraminidase gene, and the rest of the F gene.

The results obtained in this study reinforce the previous idea that the principal selective pressure resulting in the emergence of new variants of HRSV appears the immune response. Taking these results into account, it would be interesting to analyze in further studies the effect of passaging in tissue culture on the variation of other HRSV proteins, different from the attachment G protein.

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