Available online at www.sciencedirect.com
SCIENCE DIRECT®
Antiviral Research 61 (2004) 165–171
Antiviral Research
www.elsevier.com/locate/antiviral

Isolation and characterization of NMSO3-resistant mutants of respiratory syncytial virus

Kazufumi Kimura [a], Ken Ishioka [a], Koichi Hashimoto [a], Schuichi Mori [a], Tatsuo Suzutani [a], Terry L. Bowlin [b], Shiro Shigeta [a,*]

[a] *Department of Microbiology, Fukushima Medical University School of Medicine, 1, Hikarigaoka, Fukushima 960-1295, Japan*
[b] *Microbiotix, Inc., One Innovation Drive, Worcester, MA 01605, USA*

Received 23 July 2003; accepted 29 September 2003

Abstract

We obtained two mutant strains of respiratory syncytial virus (RSV) which showed resistance against NMSO3 after 15 and 33 passages, respectively, in HEp-2 cells in the presence of 6.8 μM of NMSO3. The EC_{50} values of NMSO3 for the resistant virus strains were 0.48 and 0.93 μM, that is 4.8–9.3 times higher than that of the parent strain (EC_{50} = 0.1 μM). The most resistant strain also showed resistance against heparin but was sensitive to dextran sulfate and a polyoxotungstate, PM-523. In order to determine whether the acquisition of resistance to NMSO3 was the result of the accumulation of genetic changes of virus, we sequenced the G- and F-protein genes. In comparison with the standard type of RSV strains, we identified changes of 10 amino acids in the G protein including those at the central conserved segment. However, we did not observe any particular changes in the amino acid sequence of the F-protein of the resistant strains. From these results, we conclude that NMSO3 inhibits the G-protein interaction to the receptor. The mutations in the G-protein may result in the observed phenotypic resistance of RSV towards NMSO3.

Keywords: Mutants; Syncytial virus; Phenotypic resistance

1. Introduction

NMSO3, a sulfated sialyl lipid, has been evaluated for antiviral activity against several DNA and RNA viruses. We have previously shown antiviral efficacy against respiratory syncytial virus (RSV) and rotavirus infections in vitro and in vivo and against adenovirus in vitro (Kimura et al., 2000; Takahashi et al., 2002; Kaneko et al., 2001). The median effective concentration (50% effective concentration, EC_{50}) of NMSO3 against RSV replication (standard Long strain) in HEp-2 cells was 0.2 and 0.32 μM, as monitored by optical ELISA and plaque reduction, respectively. NMSO3 showed potent anti-RSV activity against six fresh clinical isolates with an average EC_{50} value of 0.23 μM. The mechanism of anti-RSV activity of NMSO3 was assumed to be virus adsorption or penetration based upon time of addition experiments and inhibition of syncytium formation (Kimura et al., 2000). It is well established that the G glycoprotein of RSV is responsible for virus binding to the cell surface receptor and the F glycoprotein mediates the fusion of the viral and cell membranes (Levine et al., 1987; Walsh and Hruska, 1983). After these early processes, the cells allow the entrance of the virus nucleocapsid into the cytoplasm. In order to understand the mechanism of antiviral action of NMSO3-against RSV, we selected several NMSO3 resistant clones of RSV and analyzed the amino acid sequences of the G and F proteins.

2. Materials and methods

2.1. Cell cultures and viruses

HEp-2 cells were serially passaged using Eagle's minimum essential medium (EMEM) supplemented with 5% fetal calf serum (FCS), 100 units/ml of penicillin G, 100 μg/ml of streptomycin, 0.2% sodium bicarbonate and 2 mM L-glutamine. The origin of the Long strain of RSV and preparations of the virus stocks have been reported previously (Kawana et al., 1987). Virus was passaged in HEp-2 cells in maintenance medium (MM, EMEM supplemented with 2% heat inactivated fetal calf serum, 0.2% sodium

* Corresponding author. Tel.: +81-24-547-1111; fax: +81-24-547-1995.
E-mail address: sshigeta@fmu.ac.jp (S. Shigeta).

0166-3542/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2003.09.008

bicarbonate, 2 mM L-glutamine and antibiotics) and virus stock was kept at −80 °C. RSV Long strain was provided by Y. Numazaki from the National Sendai Hospital.

2.2. Chemicals and reagents

The chemical name of NMSO3 is sodium [2,2-bis (docosyl-oxymethyl)propyl-5-acetoamide-3,5-dideoxyl-4,7, 8,9-tetra-*O*-(sodium-oxysulfonyl)-D-glycero-α-D-galacto-2-nonulopyranosid]orate. NMSO3 has a molecular weight of 1478.7. The compound was synthesized at The Central Research Institute of Nisshin Food Product Co., Ltd., Kusatsu, Shiga, Japan. The structural formula of NMSO3 has been shown previously (Kimura et al., 2000). NMSO3 is soluble in water, i.e., more than 10% at 60 °C and more than 20% at 80 °C. Dextran sulfate (molecular weight 5000, DS5000) was purchased from Sigma (St. Louis, MO). Heparin was obtained from medicinal supplies (Upjohn heparin sodium injection from lung) with a molecular weight of 5000–20,000. PM-523, $(PriNH_3)_6H[PTi_2W_{10}O_{38}(O)_2]H_2O$ (where Pri is isopropanol), a Keggin type polyoxometalate, was synthesized by a procedure published elsewhere (Yamase et al., 1992). PM-523 has been fully characterized by chemical analysis, nuclear magnetic resonance and infrared spectroscopy (Yamase et al., 1992). Stock solutions of NMSO3 and DS5000 were prepared in sterile distilled water at 10 mg/ml and PM-523 was prepared in dimethyl sulfoxide (DMSO, Sigma) at 10 mg/ml. Heparin was supplied as a solution of 1000 units/ml. Additional dilutions were made in MM when the compounds were tested in cell culture.

2.3. Evaluation of sensitivity for NMSO3

Evaluation of sensitivity for NMSO3 was assessed by the plaque reduction (PR) method. The PR method for quantifying RSV sensitivity for antiviral compounds has been described previously (Shigeta et al., 1992). Briefly, 1 ml of HEp-2 cell suspension, containing 3×10^4 cells in EMEM plus 5% FCS, was seeded in each well of the culture plate (Multiwell™ 12 well, Falcon) and incubated at 37 °C. A monolayer of cells were obtained after a 24-h incubation period, and inoculated with approximately 100 plaque forming units (PFU) of RSV in 100 μl of MM. Cells were simultaneously treated with a five-fold serial dilution of antiviral compound in 100 μl of MM. The cells were incubated at 35 °C for 90 min following the inoculation of RSV. The inoculum was then aspirated and 1000 μl of MM containing the same concentration of compound and 1% methylcellulose was added to each well. After 3 days of incubation at 35 °C the medium was aspirated and cells were fixed with 5% formalin in PBS for 3 min and then stained with 0.02% crystal violet. The number of plaques were counted at 40× magnification, and the concentration of the compounds, which reduced the number of plaques to 50% of the control, was determined as the EC_{50} using probit statistics method. In order to assess the effect of the compound during the virus adsorption period, the overlay medium (MM plus 1% methylcellulose) in which antiviral compounds were absent, was used in some experiments.

2.4. Selection of NMSO3 resistant mutants

The Long strain of RSV was plaque purified and a strain designated as Long 1 was obtained from a single plaque. The Long 1 strain was then passed in HEp-2 cells in the presence of NMSO3 (starting at 1 μg/ml and gradually increased up to 10 μg/ml = 6.8 μM) until the virus showed resistance to NMSO3. Following 15 passages of Long 1 strain in the presence of NMSO3, a resistant strain (2CO) was obtained. At passage 33 under NMSO3 pressure, a second resistant virus strain (4CO) was obtained. These strains were plaque purified in the presence of 10 μg/ml of NMSO3 and were stored at −80 °C until use.

2.5. RSV-RNA extraction and RT-PCR amplification

Sequence analysis of RNA from Long 1 and the mutant strains were performed as outlined below. Viral RNA was extracted from RSV-infected HEp-2 cells using a commercial kit (QIAmp RNA Blood Mini kit, Qiagen). Reverse transcription (RT) of viral RNA to specific cDNA of the G and F genes was performed using the primers G outer *forward* and F outer *forward*, respectively. A nested polymerase chain reaction (PCR) was performed as follows: 2 μl each of extracted RNA and 1 μl of primer (Table 1) were combined in 0.5 mM dNTP mixture, first-strand buffer, 10 μM DTT, and 40 units of super script™IIRNA ase H⁻ reverse transcriptase (Invitrogen) in 10 ml of reaction volume. The mixture was incubated at 42 °C for 50 min and followed by inactivation by heating at 70 °C for 15 min. RSV G and F protein genes were amplified from the cDNAs, obtained as above, by nested PCR using the Expand High Fidelity PCR system (Roche) and the primer sets as shown in Table 1. The first set of primers G outer *forward* and G outer *reverse* were used for the first round of the nested PCR for the G protein gene and the inner set of primers, G inner *forward* and G inner *reverse* were used for the second round of the nested RCR. Similarly, the set of F outer *forward* and F outer *reverse*, and the set of F inner *forward* and F inner *reverse* were used for the F protein gene for the first round and second round of the nested PCR, respectively. The cycle parameters of the first round of PCR for the G protein gene were as follows: 30 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 120 s, with a final extension at 72 °C for 10 min. The cycle parameters of the first round of PCR for the F protein gene were as follows: 30 cycles of denaturation at 95 °C for 30 s, annealing at 47 °C for 30 s and extension at 72 °C for 150 s, with a final extension at 72 °C for 10 min. The cycle parameter of the second round of PCR for the G protein gene were as follows: 30 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 90 s, with a final exten-

Table 1
Sequences of primers and characteristics of each nested RT-PCR

Gene	Primers	Positions[a]	Sequence (5′,3′)	Length of amplified product (bp)
G	G outer for[b]	3985–4004	5′-GGAAGCACACAGCTACACGA-3′	1817
	G outer rev	5802–5777	5′-GAGCACTAAGATAGCCTTTGCTAAC-3′	
G	G inner for	4582–4607	5′-CCCACCATGCAAACCACTATCCATAC-3′	1088
	G inner rev	5670–5644	5′-GCAACTCCATTGTTATTTGCCCCAGAG-3′	
F	F outer for	5487–5511	5′-CAACTTCTCCGAAGGCAATCCAAG-3′	1969
	F outer rev	7456–7437	5′-CCAATGACAGATGGGTTGTC-3′	
F	F inner for	5592–5618	5′-TAAACTCTGGGGCAAATAACAATGGA-3′	1778
	F inner rev	7370–7347	5′-CCATTGTAAGAACATGATTAGGTGCT-3′	
F	F + 435	6082–6104	5′-TGTTAGGTGTTGGATCTGCAATC-3′	
F	F − 457	6104–6083	5′-GATTGCAGATCCAACACCTAAC-3′	
F	F + 1233	6880–6899	5′-TCACATCTCTAGGAGCCATT-3′	
F	F − 1399	6986–6967	5′-TGCATAATCACACCCGTTAG-3′	

[a] Numbers are counted from the beginning of the whole viral RNA.
[b] for: forward, rev: reverse.

sion at 72 °C for 10 min. The cycle parameter of the second round of PCR for the F protein gene were as follows: 30 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 90 s, with a final extension at 72 °C for 10 min. Amplified products of the second round of PCR were expected as 1088 base pairs (bp) and 1778 bp in sizes of the G protein gene and F protein gene, respectively, and visualized by electrophoresis on a 1% agarose gel stained with ethidium bromide. These products were purified using a commercial kit (MinElute Gel Extraction Kit Qiagen).

2.6. Analysis of nucleotide and amino acid sequences

Amplified and purified gene products were directly sequenced on the ABI PRISM^{R} 310 Genetic Analyzer (Applied Biosystems) using the ABI PRISM BigDye^{TM} Terminator V 1.1 Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). For the sequencing of the G protein genes, primers G inner *forward* and G inner *reverse* were used. For the sequencing of the F protein genes, primers F inner *forward* and F inner *reverse*, F + 435 and F − 457, F + 1233 and F − 1399 were used (see Table 1). Sequences were analyzed using SeqEd^{TM}Ver 1.0.3 and multiple sequence alignments were generated with GENETYX-SV/R Ver 5.1.1. Sequences were compared with the amino acid sequences of the reported Long strain obtained from GenBank (the accession numbers of the sequences were M17212 and M22643 for G and F protein amino acid sequences, respectively).

2.7. Statistical analysis

Statistical analysis was performed with the Statcel version 020.5 software (produced by Yanai, 1998). The results were expressed as the mean ± 1 standard error of the mean. To measure differences among groups, the results were tested for significance by a non-parametric way (Kruskal–Wallis statistics). Results having *p*-values of less than 0.05 were considered significant.

3. Results

3.1. NMSO3 resistant strains

RSV Long 1 strain was passed 33 times in the presence of NMSO3 as described above. A NMSO3 resistant strain, designated as 4CO, as well as the intermediate strain, 2CO obtained at 15 passages of RSV, were isolated. The EC_{50} of NMSO3 against the escaped mutants were evaluated. The EC_{50} of NMSO3 for the 4CO mutant was 0.93 μM, that is 9.3 times higher than that of the Long 1 strain (EC_{50} = 0.1 μM). On the other hand, the EC_{50} for 2CO was 0.48 μM, thus 4.8 times higher than that of the Long 1 strain. The 4CO mutant was passaged a further 10 times in HEp-2 cells in the presence of NMSO3, however, the EC_{50} of NMSO3 for the escaped strain did not further increase and the gene sequence of the G protein did not show additional changes after the additional 10 passages (data not shown).

3.2. Evaluation of the cross-resistance of the mutant CO4 for the other adsorption inhibitors

The 4CO strain was tested for resistance against other compounds known to inhibit binding or adsorption of RSV to cells. Heparin, DS5000 and the polyoxometalate PM-523 were added to cells in two different ways: compound was present only during the virus adsorption and then withdrawn from the overlay medium (designated as “without”) or the compound was present throughout the experiment (the same concentration of the compound during the virus adsorption was also added to the overlay medium, designated as “with”). The results showed that 4CO gained a 10-fold resistance (EC_{50} of NMSO3 for 4CO was 9–11 times higher than that for Long 1 strain). The EC_{50} value did not change whether NMSO3 was present or absent in the overlay medium after the 90 min virus adsorption period (Table 2). The 4CO strain also showed resistance against heparin. However, the resistance for heparin was somewhat

Table 2
EC_{50} of NMSO3, heparin, PM-523 and DS5000 for NMSO3-escaped mutant 4CO and its parent strain (Long 1)

Compound	Method[a]	EC_{50} (μM)		*p*
		4CO	Long 1	
NMSO3	Without	0.90 ± 0.45 (10)[b]	0.08 ± 0.10 (9)	0.0002*
	With	0.93 ± 0.60 (5)	0.1 ± 0.12 (5)	0.03*
Heparin	Without	0.06 ± 0.06 (4)[c]	0.02 ± 0.015 (6)	0.247
	With	0.11 ± 0.08 (5)	0.02 ± 0.016 (5)	0.033*
PM-523	Without	0.92 ± 0.4 (5)	0.75 ± 0.24 (5)	0.416
	With	0.49 ± 0.35 (5)	0.16 ± 0.14 (4)	0.154
DS-5000	Without	0.17 ± 0.08 (4)[c]	0.18 ± 0.09 (3)	0.88
	With	0.25 ± 0.15 (4)	0.21 ± 0.07 (5)	0.68

[a] In "without", compound was withdrawn from the overlay medium after virus adsorption. In "with", compound was kept in the overlay medium throughout the experiment.
[b] The numbers in parentheses indicate the number of experiments.
[c] Unit/ml and μg/ml were employed for heparin and DS5000, respectively.
* $P < 0.05$ is considered significant.

smaller than for NMSO3, i.e., EC_{50} of heparin for 4CO increased about 3–5.5 times, as compared to the Long 1 strain. DS5000 also showed antiviral activity against RSV, however, the 4CO strain did not show diminished sensitivity against DS5000, when compared to the Long 1 strain. PM-523 also inhibited RSV adsorption, whereas its antiviral activity was decreased when it was withdrawn from the overlay medium after the virus adsorption (Table 2). PM-523 may interfere with virus growth when added after, as well as during, virus adsorption. The EC_{50} of PM-523 for 4CO increased by 1.2–3-fold compared to that for the Long 1 strain, however, this increase was not statistically significant.

3.3. Sequence analysis of amino acids of the mutant G and F proteins

Sequences in the viral RNA sense of G and F protein genes were analyzed and the amino acids of the G and F proteins were compared among the Long 1 (parent strain), 2CO (intermittent strain) and 4CO (NMSO3 resistant strain). The sequences were also compared between genes of the standard Long strain (Gen Bank accession number M17212 for G and M22643 for F protein genes) and the amplified genes of the above mentioned strains. In the G protein, three amino acid differences were observed between Long (M17212) and Long 1. Among Long 1 and 2CO, six amino acids changes were observed (F163P, F165L, F170S, I189T, L215P, and L274P). When Long 1 was compared with 4CO, four more amino acid mutations were added to 2CO (L97P, F101L, I107T and I114T). In addition, F168S and F265L were found instead of F165L and L215P, observed in 2CO (Table 3). As mentioned above, the 2CO and 4CO strains were mutants which were obtained by 15 and 33 passages of Long 1 strain, in HEp-2 cells, in the presence of NMSO3, respectively. The mutation of the amino acid sequence in the G protein occurred at first between 163 and 189 and then extended from 97 to 114 (Table 3, Fig. 1). In Fig. 1, the mutation points of amino acids in G protein of 4CO strain is illustrated. Four of the 10 mutation points located near the conserved domain (amino acids 164–176) and six were outside of the conserved domain. In the next experiment, the amino acid sequence of the F protein was analyzed and changes were observed between Long and Long 1 at 7 positions (data not shown). Only one change was observed, at position 210 (K to E), between Long 1 and 4CO. However, one more nucleotide change, which did not result in an amino acid change, was noted at position 638 (A to G).

Table 3
Mutation points of amino acids and nucleotides in G protein (gene) of NMSO3-escaped mutants 2CO and 4CO

Strains	2CO	4CO	Long 1
EC_{50} for NMSO3 (μM)	0.48	0.93	0.1
No. of the changed a.a.[a]	6	10	0
Sorts and positions[a]		L97P (290)	
		F101L (301)	
		I107T (320)	
		I114T (341)	
	F163P (487, 488)	F163L (487)	
	F165L (493)		
		F168S (503, 504)	
	F170S (509)	F170S	
	I189T (566)	I189T	
	L215P (644)		
		F265L (793)	
	L274P (831)	L274P	

Numbers in parentheses indicate sequence number of nucleotide which changed in the mutants. All nucleotide changes were U to C.
[a] Change from Long 1 strain.

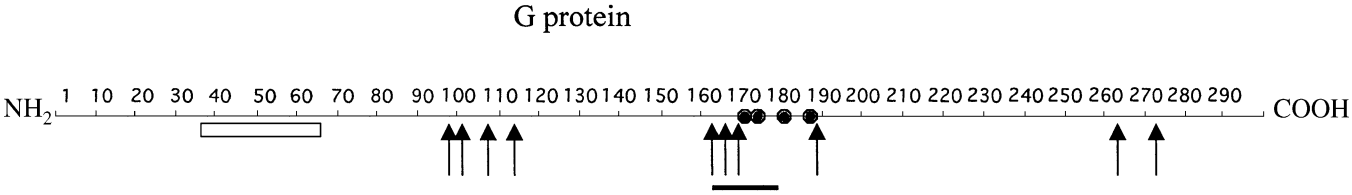

Fig. 1. Amino acid sequence changes in G protein of the NMSO3-resistant RSV 4CO strain. Diagram of the G protein primary structure indicating the transmembrane region (open rectangle line) and the cystein residues (closed circles). Conserved domains (amino acids 164–176) are indicated by the horizontal bar under the diagram. Locations of the sequence changes are indicated by arrows. Amino acid numbers are counted from the beginning of the G protein.

4. Discussion

Thirty-three passages in the presence of NMSO3 were needed to obtain a resistant strain against NMSO3, thus considerably more passages compared with previous reports in which the resistant RSVs for antiviral substances were selected by passages in the presence of the inhibitors. Escape mutants from the anti-RSV monoclonal antibodies (Mabs) were obtained after 5–10 passages of RSV in the presence of Mabs. These mutants lost reactivity with the Mabs (Martinez et al., 1997). Sudo et al. (2001) reported that a resistant mutant to RD3-0028 which was obtained after 18 passages in the presence of the antiviral compound. The mutant strain was 80-fold less susceptible to RD3-0028 than the parent Long strain. In this study, RSV acquired resistance for NMSO3 after 33 passages of the susceptible RSV in the presence of NMSO3, and showed only 10-fold higher EC_{50} than the EC_{50} of the original strain. From these results it can be concluded that it is difficult for RSV to acquire resistance against NMSO3.

Heparin, dextran sulfate and PM-523 were reported to inhibit early stages of RSV infection, such as virus adsorption to and penetration through the cell membrane (Hosoya et al., 1991; Bourgeois et al., 1998; Shigeta et al., 1996). Based upon previous results, the mechanism of anti-RSV activity of NMSO3 was presumed to be inhibition of adsorption to cells, penetration through the cell membrane and syncytium formation (Kimura et al., 2000). The NMSO3-resistant strain 4CO showed resistance against heparin, although the level of resistance was smaller than for NMSO3. Between Long 1 and 4CO, the *p*-values for the difference of sensitivity to NMSO3 or heparin were less than 0.03 (when compounds were continued to be present throughout the experiment). On the other hand, 4CO did not show significant resistance against DS5000 or PM-523 compared with Long 1 strain (*p*-values were from 0.15 to 0.68 when compounds were present through the experiment). From these results, we can conclude that there may be some similarity in mechanism by which NMSO3 and heparin inhibit the infection of RSV.

Heparin binding proteins are known to interact with heparin via electrostatic charge interactions generated between the negatively charged sulfate groups on heparin and the positively charged amino acids within the proteins in the heparin binding domain (HBD) (Flynn and Ryan, 1995). The ectodomain of the RSV-G protein contains a cluster of positively charged amino acids (180P to 233K) which was postulated to be RSV-G heparin binding ectodomains (Krusat and Streckert, 1997). Two lineal sequences of RSV ectodomains were identified from the G proteins of RSV types A and B, respectively, as important domains for the binding of RSV to heparin-containing receptors. The peptides were ^{184}AICKRIPNKKPGKKT^{198} for type A and ^{183}KSICKTIPSNKPKKK^{197} for type B (Feldman et al., 1999). On the other hand, several workers have recently reported that the G glycoprotein of RSV is not indispensable for the growth of RSV in Vero cells. A cold adapted RSV-B mutant *cp-52* was found to be spontaneously deleted in the SH and G genes (Karron et al., 1997). A mutant recombinant of RSV of which G gene was artificially truncated and deleted was created (Teng et al., 2001; Teng and Collins, 2002). Both mutant RSVs were reported to grow as efficiently as wild type RSV in Vero cells but did not grow efficiently in HEp-2 cells.

Changes of amino acids in the G protein of 4CO from its parent strain (Long 1) are described in Table 3 and Fig. 1. The 4CO mutant underwent 10 amino acids changes through 33 passages in the presence of NMSO3. Among them, amino acid changes I107T, I114T, and L274P could be found among the virus isolates during 1956–1993 in United States and Europe (Cane and Pringle, 1995). F265L and L274P were also commonly found in Mozambique wild strains (Roca et al., 2001). On the other hand, amino acid changes L97P, F101L, F163L, F168S, F170S, I189T were not found in the reported wild type strains. These changes in the G protein of the NMSO3-resistant 4CO strain are mostly located in the central conserved segment of the G ectodomain. It was reported that a stretch of 13 amino acids (position 164–176 in the amino acid sequence of the G protein) was conserved in the same form in several different strains and subgroups of RSV and this segment has been proposed as the receptor binding site and includes a cluster of four cysteine residues (Johnson et al., 1987). This region overlaps with four closely spaced cysteine residues (positions 173, 176, 182 and 186) that form disulfide bonds in the patterns 1–4 and 2–3 and create a cysteine noose (Melero et al., 1997).

Point mutations in the conserved region of the G protein were also reported in Mab-resistant RSV and residues

F163 and I189 were substituted by different amino acids (Martinez et al., 1997). This indicates that even the amino acid sequence in the conserved domain can be changed when the antiviral substance works on this location and then resistant mutants escape the action of inhibitors. Therefore, it can be deduced that NMSO3 probably interacts with the central conserved segment of G protein ectodomain and mutations in amino acids in this region may partially account for the antiviral resistance of NMSO3. In above mentioned reports by Teng et al. (2001) and Teng and Collins (2002), they created additional recombinant RSVs in which the major parts of HBD were deleted, that is, amino acids 167–181 and 187–197. These recombinant RSVs did not lose their efficiency of growth in HEp-2 as well as Vero cells and still proved sensitive to soluble heparin. Thus, HBD of G protein may not be indispensable for the binding of RSV to heparin-containing receptors.

More recently, the G protein of RSV was shown to be a mimic of fractalkine, a proinflammatory CX3C chemokine. The G protein appears to be able to bind to the fractalkine receptor CX3CR1 (Tripp et al., 2001). Another group isolated annexin II as a potential RSV receptor from HEp-2 cells (Malhotra et al., 2003). Considering these reports, it is conceivable that G protein may also interact with different receptors from heparin-like glycosaminoglycan using other portion of the molecule. In comparison to the mutation points that appeared in the G protein of the 2CO strain, the 4CO strain acquired additional mutations in the amino acids from L98 to I114 (Table 3). Since the antiviral activity of NMSO3 decreased against 4CO, compared with 2CO, the mutations of amino acids located outside of the conserved G region may also support and enhance the resistance to NMSO3. As indicated in Table 3, among 10 amino acids which mutated after selection with NMSO3 treatment, 7 were changed in the residues from “hydrophobic” to “hydrophilic”. These changes in the residues and electrostatic charge of amino acids may influence the interaction between the G protein and its receptor as well as its interaction with the inhibitors.

In the previous paper, we reported that NMSO3 inhibited the adsorption of RSV to HEp-2 cells and the penetration through the cell membrane (Kimura et al., 2000). When we examined the inhibition of adsorption and penetration with stepwise diluted concentrations of several inhibitors, the pattern of inhibition of NMSO3 was quite similar to that observed for anti-F Mab and different from that observed by DS5000. Thus, we presumed that NMSO3 seemed to associate with the F-protein of RSV to express its anti-RSV activity. Therefore, we analyzed the amino acid sequence of the F-protein of both susceptible and resistant strains of RSV. A mutation of a single amino acid in the F-protein of 4CO was observed only at K210E. A mutation in this position is not specific for the resistant strain and can be observed among wild type strains (Gen Bank accession no. 056863 is different at K210Q from the Long strain). In conclusion, the NMSO3 resistant strain 4CO had several mutations in amino acid sequence at in- and outside of the conserved region of the G protein. These positions are apparently involved in the antiviral action of NMSO3 against RSV.

References

Bourgeois, C., Bour, J.B., Lidholt, K., Gauthray, C., Pothier, P., 1998. Heparin-like structures on respiratory syncytial virus are involved in its infectivity in vitro. J. Virol. 72, 7221–7227.

Cane, P.A., Pringle, C.R., 1995. Evolution of subgroup A respiratory syncytial virus: evidence for progressive accumulation of amino acid changes in the attachment protein. J. Virol. 69, 2918–2925.

Feldman, S.A., Hendry, R.M., Beeler, J.A., 1999. Identification of a linear heparin binding domain for human respiratory syncytial virus attachment glycoprotein G. J. Virol. 73, 6610–6617.

Flynn, S.J., Ryan, P., 1995. A heterologous heparin-binding domain can promote functional attachment of a pseudorabies virus gC mutant to cell surfaces. J. Virol. 69, 834–839.

Hosoya, M., Balzarini, J., Shigeta, S., De Clercq, E., 1991. Differential inhibitory effects of sulfated polysaccharides and polymers on the replication of various myxoviruses and retroviruses, depending on the composition of the target amino acid sequences of the viral envelope glycoproteins. Antimicrob. Agents Chemother. 35, 2515–2520.

Johnson, P.R., Spriggs, M.K., Olmsted, R.A., Collins, P.L., 1987. The G glycoprotein of human respiratory syncytial viruses of subgroups A and B: extensive sequence divergence between antigenically related proteins. Proc. Natl. Acad. Sci. U.S.A. 84, 5625–5629.

Kaneko, H., Kato, K., Mori, S., Shigeta, S., 2001. Antiviral activity of NMSO3 against adenovirus in vitro. Antivir. Res. 52, 281–288.

Karron, R.A., Buonagurio, D.A., Georgiu, A.F., Whitehead, S.S., Adams, J.E., Clements-Mann, M.L., Harris, D.O., Randolph, V.B., Udem, S.A., Murphy, B.R., Sidhu, M.S., 1997. Respiratory syncytial virus (RSV) SH and G proteins are not essential for viral replication in vitro: clinical evaluation and molecular characterization of a cold-passaged, attenuated RSV subgroup B mutant. Proc. Natl. Acad. Sci. U.S.A. 94, 13961–13966.

Kawana, F., Shigeta, S., Hosoya, M., Suzuki, H., De Clercq, E., 1987. Inhibitory effects of antiviral compounds on respiratory syncytial virus replication in vitro. Antimicrob. Agents Chemother. 31, 1225–1230.

Kimura, K., Mori, S., Tomita, K., Ohno, K., Takahashi, K., Shigeta, S., Terada, M., 2000. Antiviral activity of NMSO3 against respiratory syncytial virus infection in vitro. Antivir. Res. 47, 41–51.

Krusat, T., Streckert, H.J., 1997. Heparin-dependent attachment of respiratory syncytial virus (RSV) to host cells. Arch. Virol. 142, 1247–1254.

Levine, S., Klaiber-Franco, R., Paradiso, P.R., 1987. Demonstration that glycoprotein G is the attachment protein of respiratory syncytial virus. J. Gen. Virol. 68, 2521–2524.

Malhotra, R., Ward, M., Bright, H., Priest, R., Foster, M.R., Hurle, M., Blair, E., Bird, M., 2003. Isolation and characterisation of potential respiratory syncytial virus receptor(s) on epithelial cells. Microbes Infect. 5, 123–133.

Martinez, I., Dopazo, J., Melero, J.A., 1997. Antigenic structure of the human respiratory syncytial virus G glycoprotein and relevance of hypermutation events for the generation of antigenic variants. J. Gen. Virol. 78, 2419–2429.

Melero, J.A., Garcia-Barreno, B., Martinez, I., Pringle, C.R., Cane, P.A., 1997. Antigenic structure, evolution and immunobiology of human respiratory syncytial virus attachment (G) protein. J. Gen. Virol. 78, 2411–2418.

Roca, A., Loscertales, M.-P., Quinto, L., Perez-Brena, P., Vaz, N., Alonso, P.-L., Saiz, J.-C., 2001. Genetic variability among group A and B respiratory syncytial viruses in Mozambique: identification of a new cluster of group B isolates. J. Gen. Virol. 82, 103–111.

Shigeta, S., Mori, S., Baba, M., Honzumi, K., Nakamura, K., Oshitani, H., Numazaki, Y., Matsuda, A., Obara, T., Shuto, S.,

De Clercq, E., 1992. Antiviral activities of ribavirin, 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxiamide, and 6′-(*R*)-*C*-methyl-neplanocin A against several ortho- and paramyxoviruses. Antimicrob. Agents Chemother. 36, 435–439.

Shigeta, S., Mori, S., Watanabe, J., Yamase, T., Schinazi, R.F., 1996. In vitro anti-myxovirus activity and mechanism of anti-influenza virus activity of polyoxometalates PM-504 and PM-523. Antivir. Chem. Chemother. 7, 346–352.

Sudo, K., Konno, K., Watanabe, W., Shigeta, S., Yokota, T., 2001. Mechanism of selective inhibition of respiratory syncytial virus by a benzodithiin compound (RD3-0028). Microbiol. Immunol. 45, 531–537.

Takahashi, K., Ohashi, K., Abe, Y., Mori, S., Taniguchi, K., Ebina, T., Nakagomi, O., Terada, M., Shigeta, S., 2002. Protective efficacy of a sulfated sialyl lipid (NMSO3) against human rotavirus-induced diarrhea in a mouse model. Antimicrob. Agents Chemother. 46, 420–424.

Teng, M.N., Collins, P.L., 2002. The central conserved cystine noose of the attachment G protein of human respiratory syncytial virus is not required for efficient viral infection in vitro or in vivo. J. Virol. 76, 6164–6171.

Teng, M.N., Whitehead, S.S., Collins, P.L., 2001. Contribution of the respiratory syncytial virus G glycoprotein and its secreted and membrane-bound forms to virus replication in vitro and in vivo. Virology 289, 283–296.

Tripp, R.A., Jones, L.P., Haynes, L.M., Zheng, H., Murphy, P.M., Anderson, L.J., 2001. CX3C chemokine mimicry by respiratory syncytial virus G glycoprotein. Nature Immunol. 2, 732–738.

Walsh, E.E., Hruska, J., 1983. Monoclonal antibodies to respiratory syncytial virus proteins: identification of the fusion protein. J. Virol. 47, 171–177.

Yamase, T., Ozeki, T., Motomura, S., 1992. 183 W NMR and X-ray crystallographic studies on the peroxo complexes of the Ti substituted α-Keggin typed synergistic tungstophosphates. Bull. Chem. Soc. Japan 65, 1453–1459.

Yanai, H., 1998. 4 steps excel statistics. OMS Publications, Tokyo, Japan.