



NeuAc α 2,3Gal-Glycoconjugate Expression Determines Cell Susceptibility to the Porcine Rubulavirus LPMV

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ABSTRACT. Relevance of membrane sialoglycoconjugates as receptors for infection by the porcine rubulavirus has been determined *in vitro* by sugar and lectin competition assays and by inhibition of glycosylation. Our results show that NeuAc α 2,3Gal but not NeuAc α 2,6Gal inhibits the virus infectivity of Vero cells, and the virus was effectively blocked with the lectin *Maackia amurensis*, specific for NeuAc α 2,3Gal. Inhibition of the cellular glycosylation with tunicamycin, deoxinojirimycin as well as neuraminidase treatment diminishes the viral capacity to bind and infect this cell line. Dexamethasone, which promotes the activity of sialyl α 2,6 glycosyltransferase, also diminishes the cell susceptibility for infection. This is the first report confirming that NeuAc α -2,3Gal recognition is determinant in the pathogenesis of the porcine rubulavirus. COMP BIOCHEM PHYSIOL 118B; 2:327–332, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. Sialic acid, virus receptors, lectin cytochemistry, glycosylation inhibitors, dexamethasone, paramyxovirus, glycoproteins

INTRODUCTION

The virus “La Piedad Michoacan” (LPMV) is the etiologic agent of the “blue eyes” disease of swine (12), which is characterized by neurological, reproductive and respiratory disorders and by corneal opacity (22). LPMV is a single-stranded negative sense RNA virus, which shares high homology regarding nucleotide and amino acid sequences with mumps virus, simian virus 5 and parainfluenza viruses 2 and 4 (8); hence, it has been classified as the porcine species in the genus *Rubulavirus*, subfamily *Paramyxovirinae*, family *Paramyxoviridae*, order *Mononegavirales* (14).

The first step in the virus infection process is the recognition of cellular structures that act as specific receptors. This activity determines the virus tissue tropism and is performed by viral adhesion proteins (23). In Paramyxovirus, recognition of cell receptors is accomplished by the hemagglutinin-neuraminidase (HN) envelope glycoprotein. This interaction is followed by virus-cell adhesion, membrane fusion and intracytoplasmic nucleocapsid release (5). Membrane fusion is performed by the fusogenic (F) envelope glycoprotein; however, HN plays a relevant role in the F glycoprotein-induced syncytia forming (SF) activity (1,13). We have previously shown that LPMV’s HN glycoprotein specifically recognizes sialic acid-containing structures, which inhibited the virus hemagglutinating activity (18).

Neuraminic acids (NeuAc) are ubiquitous molecules that play important roles in cell biology. They bind to galactose moieties in the oligosaccharide chain of glycoproteins or glycolipids by α 2,3 or α 2,6 glycosidic linkages. These isomeric forms of sialic acid are broadly expressed in animal cells and their expression seems to be developmentally regulated (20). In this work, we performed a series of experimental strategies to know whether sialyl α 2,3Gal- or sialyl α 2,6Gal-glycoconjugates are constitutive components of Vero cell membranes and their relevance in the porcine rubulavirus infection process.

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MATERIALS AND METHODS

Reagents

Neuraminidase (from *Vibrium cholera*), bovine trypsin, Hank’s balanced salt solution, May-Grünwald and Giemsa (MGG) stain, fluorescein isothiocyanate (FITC), dimethylsulfoxide, *Arachis hypogaea* (PNA) and *Canavalia ensiformis* (CON A) lectins, sugars, glycosides and glycoproteins used in this study were purchased from Sigma Chemical, (St. Louis, MO, U.S.A.). The glycosylation inhibitors tunicamycin, deoxinojirimycin and neuraminidase were purchased from Calbiochem (La Jolla, CA, U.S.A.).

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Received 25 January 1997; revised 21 April 1997; accepted 12 May 1997.

camycin (TUN), deoximannojirimycin (DMM) and deoxinujirimycin (DNM) and the lectins *Maackia amurensis* (MAA) specific for NeuAc α 2,3Gal (4) and *Sambucus nigra* (SNA) specific for NeuAc α 2,6Gal (21) were purchased from Boehringer-Mannheim Biochemica (Mannheim, Germany). Dexamethasone was from Pfizer (Mexico). High purity NeuAc α 2,6 and α 2,3 lactose were a kind gift of Dr. Jean Claude Michalsky from the Université des Sciences et Technologies de Lille, France.

Cells and Virus

African green monkey kidney Vero cells and pig kidney PK-15 cells were maintained in Eagle's minimal essential medium (EMEM) supplemented with 100 μ g/ml L-glutamine and 10% fetal bovine serum (FBS). The porcine LPMV rubulavirus was replicated in PK-15 cells, clarified at low speed and concentrated by centrifugation at 100,000 g, 16 hr at 4°C (12).

Virus Infectivity

All experiments were developed in Vero cell line monolayers cultured with EMEM and 2% FBS over coverslips in 96-well microassay plates, except when specified. LPMV was invariably used in each assay at 1000 SF units/ml (SFU/ml). After 96 hr of incubation at 37°C, infected cells were fixed with 70% methanol and stained with MGG stain to quantify the virus-induced syncytia formation. Virus infectivity has been determined by the number of syncytia (one syncytium = 10 or more fused cells) founded in 10 visual fields (18). Results represent three independent observations, and \pm SE of the mean was never higher than 10%.

Sugar-Cell Receptor Competition Assays

LPMV was incubated in hr at 4°C with serial dilutions (starting concentration 200 mM) of NeuAc, NeuAc α 2,3- or α 2,6-lactose or other cationic and anionic glycosides, sugars and glycoproteins; 100 μ l of virus-sugar mixtures were applied on Vero cell monolayers, incubated 2 hr at 37°C. Cells were then washed once with Hank's solution, fresh EMEM was added and they were maintained in incubation at 37°C during 96 hr. The cell monolayers were finally stained with MGG to determine virus infectivity (10).

Cell Enzyme Treatment

Confluent Vero cell monolayers were treated with 2 U/ml neuraminidase 30 min at 37°C or with 100 μ g/ml trypsin and incubated during 30 min at 37°C. Treated cells were then washed with Hank's solution and processed to determine virus infectivity (10).

Effect of Glycosylation Inhibitors on Virus Infectivity

In an attempt to confirm the relevance of glycans as the virus cell receptor, we used glycosylation inhibitors. Vero cell monolayers in logarithmic growth phase were suspended by trypsin digestion and seeded in microassay plates. The cells were treated with serial dilutions (starting concentration, 5 μ g/ml) of TUN, DMM or DNM inhibitors, previously dissolved in EMEM. After 8 hr of incubation at 37°C, cells were washed with Hank's solution and processed to determine virus infectivity (16). The effect of the glycosylation inhibitors was confirmed by lectin cytochemistry (see below).

Effect of Dexamethasone on Virus Infectivity

Vero cell monolayers in logarithmic growth phase were suspended by trypsin digestion and seeded in microassay plates. At that moment, cells were treated with serially diluted dexamethasone (starting concentration 10 μ M). After 12 hr of incubation at 37°C, cells were washed with Hank's solution and processed to determine virus infectivity (24).

Lectin Cytochemistry

FITC-conjugated lectins were prepared according to methods previously described (7). Vero cell monolayers cultured on coverslips (untreated or previously treated with neuraminidase, trypsin, DMM, DNM or dexamethasone) were removed from microassay plates and fixed in chilled acetone 10 min and blocked with phosphate-buffered saline solution, pH 7.2, with 0.4% bovine serum albumin (PBS-BSA) for 20 min at 4°C. The cell samples were incubated with 10 μ g/ml FITC-conjugated MAA, SNA, PNA and CON A lectins during 30 min at 37°C and then were washed with 0.001% Tween 20 in PBS-BSA (7). Coverslips were mounted on PBS-glycerol (v/v) over glass slides and observed at a standard 14 Zeiss microscope equipped with an IV/2 epifluorescence condenser. Specificity controls were performed using non-conjugated lectin at the same protein concentration range as the first layer reagents. Coverslips treated in this way were always negative.

Lectin-Virus Competition Assays

Confluent normal Vero cell monolayers were incubated with serial dilutions of MAA and SNA lectins (starting concentration 100 μ g/ml) during 30 min at 15°C before addition of the virus; cells were washed twice with Hank's solution and processed to determine virus infectivity as mentioned above.

RESULTS

In this work, we performed a series of experimental strategies using African green monkey kidney Vero cells as a

model to know whether membrane NeuAc α 2,3Gal or NeuAc α 2,6Gal linked glycoconjugates are relevant in the infection induced by the porcine rubulavirus and to confirm the participation of the HN protein in the pathogenesis of this disease. SF was considered the result of porcine rubulavirus infection on Vero cells (Fig. 1A). The aim of this work was to inhibit this effect by means of sugars, glycosides, glycoproteins or lectins or by modifying the normal glycosylation pattern with enzymes or dexamethasone.

Sugar Inhibition Assays

By using different sugars, glycans and glycoproteins, we established the participation of carbohydrates as determinants in the virus infectivity. From the compounds used, 200 mM NeuAc or 20 mM NeuAc α 2,3 lactose inhibited the formation of syncytia (Figs 1B and 2). Similar results were obtained with either 0.1 μ m human IgA or α ₁-acid glycoprotein, which possess also NeuAc α 2,3Gal; α 2,6 sialyl lactose,

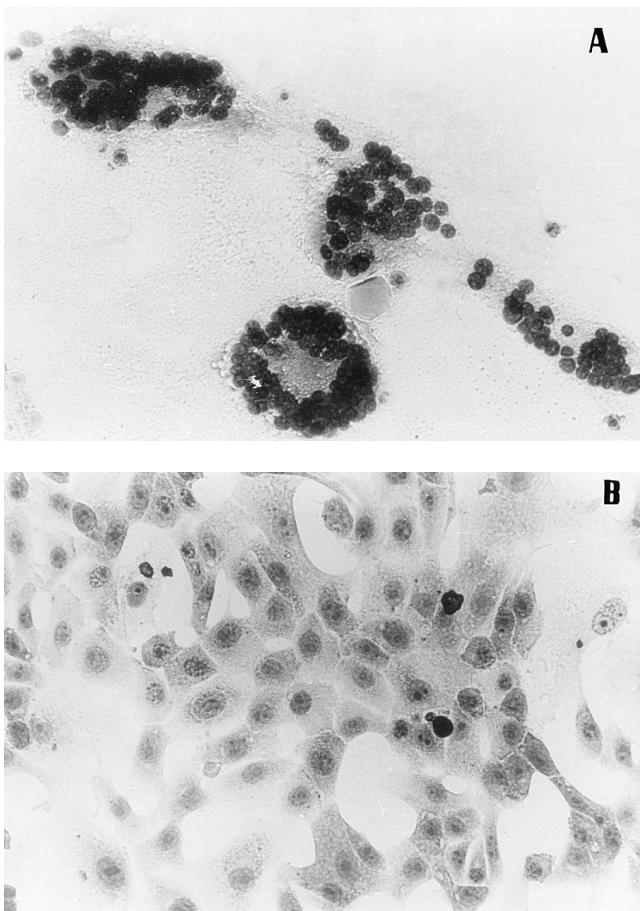


FIG. 1. Syncytia formation (considered as virus infectivity) by 1000 SFU of the porcine rubulavirus (LPMV) on Vero cell monolayers (A) and inhibition of syncytia formation of the porcine rubulavirus incubated 30 min with 20 mM NeuAc α 2,3 lactose before addition to cell monolayers (B). Magnification (A) 400 \times and (B) 600 \times .

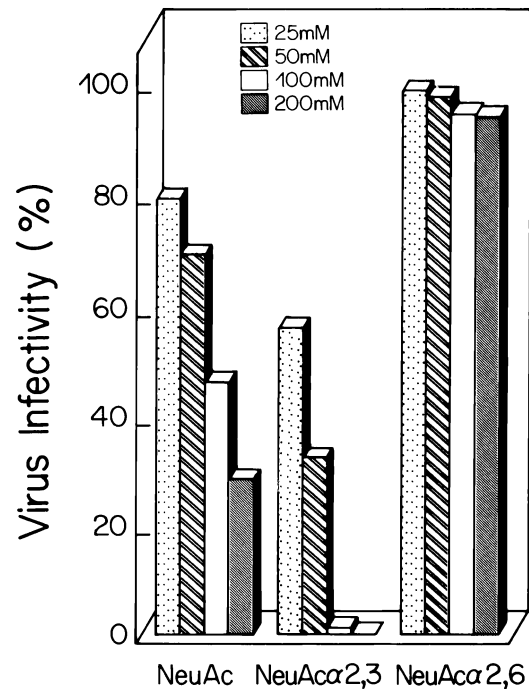


FIG. 2. Inhibition of virus infectivity with sugars. The LPMV (1000 SFU/ml) was incubated 1 hr at 4 $^{\circ}$ C with sugars or glycosides before infecting Vero cell monolayers. Only NeuAc and NeuAc α 2,3Gal-containing structures blocked the virus infectivity; 200 mM of D-galactose, D-mannose, D-glucose, L and D-fucose, N-acetyl-D-galactosamine, N-acetyl-D-glycosamine, lactose or 10 mM protamine, chondroitin and heparan sulphate were ineffective to inhibit the virus infectivity. The virus infectivity has been determined by the number of syncytia (one syncytium = 10 or more fused cells) found in 10 visual fields. Reported results represent three independent observations; \pm SE of the mean was never higher than 10%.

neutral sugars or desialylated IgA and α ₁-acid glycoprotein were ineffective to inhibit the formation of syncytia even at 200 mM (sugars) or 10 μ M (asialoglycoproteins). To identify the participation of charged groups in the interaction with NeuAc, we also used 10 mM of anionic and cationic glycosides such as protamine, heparan and chondroitin sulfate; however, these glycosides did not induce any modification in the formation of syncytia on Vero cell monolayers (not shown).

Enzyme Treatment

To confirm that NeuAc expression on Vero cell membrane is required for LPMV infection, we treated Vero cells with neuraminidase and trypsin. Sialic acid release, due to the effect of the 2 U/ml *V. cholera* neuraminidase, completely abolished the virus infectivity; trypsin treatment of the cell layers increased 200% the infectivity rate of LPMV (not shown).

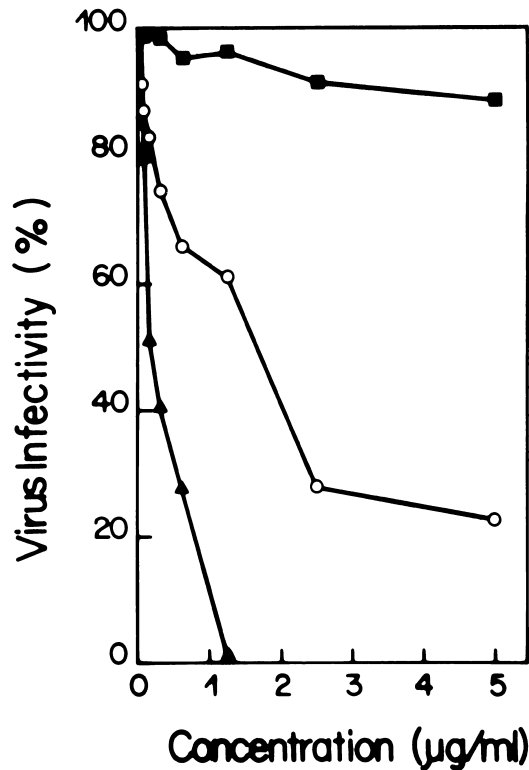


FIG. 3. Effect of glycosylation inhibitors on LPMV infectivity. Vero cell monolayers were incubated 4 hr with different glycosylation inhibitors at 5 µg/ml concentration, tunicamycin (▲—▲), deoxyojiromycin (■—■) and deoxymannojirimycin (○—○), before being infected with 1000 SFU of the porcine rubulavirus.

Effect of Glycosylation Inhibitors on LPMV Infectivity

To establish the participation of glycosylated structures as target determinants for the virus, we tried to interfere with the normal glycosylation pattern by reducing the expression of complex oligosaccharides, so we treated similar Vero cell monolayers with glycosylation inhibitors. As shown in Fig. 3, TUN completely inhibited the virus infectivity at concentrations as low as 1.5 µg/ml after 1 hr of cellular incubation, whereas DNM at 5 µg/ml diminished 75% LPMV infectivity. No significant decrease in infection was observed on DMM-treated Vero cells at the same 5 µg/ml dose after 4 hr of treatment.

Effect of Dexamethasone on LPMV Infectivity

Dexamethasone diminishes the virus infectivity in Vero cells, because 1–5 µM concentrations induce 60% inhibition of the virus infectivity (Fig. 4) after 12 hr of dexamethasone treatment. Soluble dexamethasone was shown to be a very active reagent because concentrations equal or above 10 µg/ml produced rounding, vacuolation and release of Vero cell monolayers (not shown).

Lectin Cytochemistry

We used a panel of four FITC-conjugated lectins with well-defined sugar specificity to correlate the sugar expression pattern with the susceptibility to LPMV infection and to confirm modification of glycosylation of membrane receptors. Lectins used were PNA, specific for galactose β1,3-N-acetyl-D-galactosamine; MAA, which recognizes NeuAcα-2,3Gal; SNA, which binds to NeuAc2,6αGal, and CON A, which binds oligomannosidic structures. Lectin cytochemistry indicated that Vero cells possess NeuAc linked to Gal in both α2,3 and α2,6 and low levels of desialylated Galβ1,3GalNAc and oligomannosidic structures (Table 1). After neuraminidase treatment of the Vero cells, interaction with SNA and MAA was abolished; inversely, PNA and CON A binding increased 100% when compared with normal cells (Table 1). Lectin binding assays with cells treated with glycosylation inhibitors showed that sugar expression can be completely inhibited with TUN. The binding of the four lectins was notably diminished after 4 hr of treatment and was completely absent after 8 hr of treatment. DNM treatment produced 70% reduction in positive interaction with MAA and CON A and a complete inhibi-

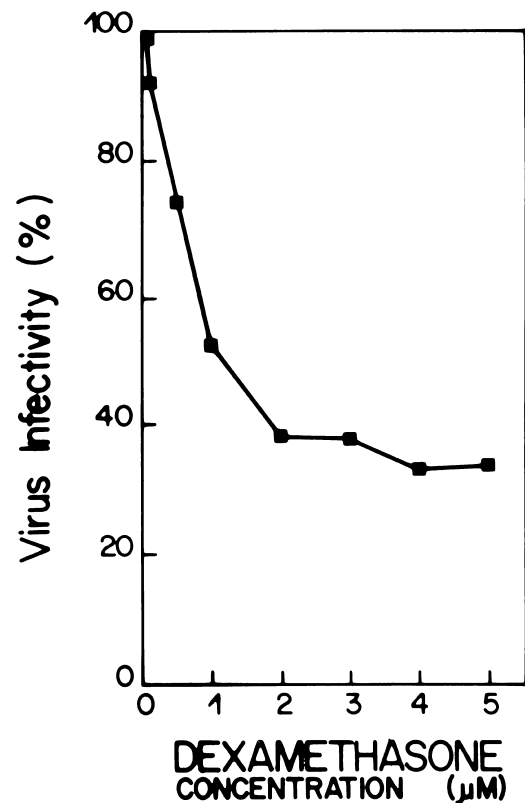


FIG. 4. Effect of dexamethasone on virus infectivity. Vero cell monolayers were treated 12 hr with serial dilutions of dexamethasone before being infected with the porcine rubulavirus. The over-expression of NeuAca2,6Gal-containing oligosaccharides reduced virus infectivity (see Materials and Methods).

TABLE 1. Lectin binding pattern in Vero cells treated with enzymes, glycosylation inhibitors and dexamethasone

	Concentration*	Lectin			
		MAA	SNA	PNA	CON A
Untreated cells		+++	++	++	++
Neuraminidase	2 U/ml	–	–	++++	++++
Trypsin	100 μ g/ml	+++	++	++	++
Tunicamycin	2 μ g/ml	–	–	–	\pm
Deoxinojirimycin	5 μ g/ml	+	–	+++	\pm
Deoximannojirimycin	5 μ g/ml	+	+	++	+
Dexamethasone	5 μ M	+	++++	–	++

Intensity of positive reactions to *Maackia amurensis* (MAA, specific for NeuAc α 2,3 Gal), *Sambucus nigra* (SNA, NeuAc α 2,6Gal), *Arachis hypogaea* (PNA, Gal β 1,3 GalNAc) and *Canavalia ensiformis* (CON A, Glc/Man) lectins are shown. Cytochemical assays were performed using 10 μ g/ml of FITC-labeled lectins.

*Optimal concentration of inhibitors.

tion of SNA reaction; however, PNA receptors increased 50% compared with normal cells. Treatment of Vero cells with DMM affects SNA, MAA and CON A lectin interaction without influencing PNA binding. Dexamethasone treatment increases 100% the cellular capacity to interact with SNA and induces a 70% inhibition on MAA binding. CON A lectin interaction remained unaltered; however, this treatment abolished interaction of PNA as compared with normal cells (Table 1).

Inhibition of Infectivity with Lectins

Incubation of normal Vero cells with MAA at 100 μ g/ml lectin concentration before adding LPMV induced 35% reduction in syncytia formation. Incubation of Vero cells with SNA, PNA or CON A did not affect LPMV infectivity (not shown).

DISCUSSION

Natural outbreaks of the porcine rubulavirus are characterized by a neurological syndrome that affects mainly newborn pigs; previous reports established that exclusively less than 20-day-old pigs are susceptible to LPMV and develop central nervous system infection (22). Host restriction is a property of most viruses, and it seems to be due to the presence of viral adhesion proteins that specifically recognize and interact with cell receptors (23). In a recent work, we identified that sialic acid-containing structures inhibit the LPMV hemagglutinating activity, suggesting their participation as specific ligands for the virus (18). Therefore, in this work we analyzed the expression of sialic acids in a susceptible cell line to understand the molecular basis of LPMV host tropism. The relevance of sialic acid was initially corroborated by neuraminidase digestion assays. Release of NeuAc from the cell membrane by neuraminidase inhibits virus infectivity, indicating that the sugar combining site for HN from LPMV is a complex sialylated struc-

ture. Moreover, trypsin treatment of the cell increases virus infectivity, indicating that the topographic modification induced by this treatment enables HN-cell surface interaction and suggests that the sialic acids that act as LPMV virus receptors are associated with cryptic receptors, such as sialyl T or sialyl Tn antigens, containing NeuAc α 2,3Gal β 1,3-GalNAc or NeuAc α 2,3GalNAc, respectively (19).

In an attempt to confirm the relevance of glycans as the virus cell receptor, we used glycosylation inhibitors, TUN, DNM and DMM (16), which interfere with the oligosaccharide transporter Dolichol phosphate, the rough endoplasmic reticulum glucosidase and the Golgi's mannosidase I, respectively (6). Our results showed that blocking early phases of the glycosylation pathway with TUN inhibits the expression of oligosaccharides able to be recognized by the porcine rubulavirus; however, treatment with DNM and DMM induced important, but not complete, inhibition of the infectivity. This conservation in the virus–cell interaction could be due to the fact that sialyl T or sialyl Tn antigens are still present despite this treatment (17). Indeed, as we demonstrated, sialylated structures are present in Vero cells after DNM or DMM treatment by interaction with the lectin MAA and not with PNA, which recognizes desialylated T or Tn antigens (9). Dexamethasone has the capacity to regulate β -galactoside- α 2,6 sialyltransferase expression (24). Our results show that dexamethasone treatment of Vero cells induces over-expression of NeuAc α 2,6Gal while seemingly decreasing NeuAc α 2,3Gal expression in cell membranes and, hence, inducing inhibition of virus infectivity. Our results indicate that Vero membrane expresses both NeuAc α 2,3Gal and NeuAc α 2,6Gal structures, confirmed by lectin cytochemistry, and that Vero cells have proved to be a useful tool in the study of virus–cell interaction.

At the present, only one sialoglycoprotein has been identified as receptor for a paramyxovirus, the human leukocyte CD46 molecule, which is recognized by the measles virus (15). Sialylated gangliosides have been demonstrated to be

receptors for some paramyxoviruses (3,5,11) and the oligosaccharide NeuAc α 2,3Gal β 1,3GalNAc (Sialyl T antigen) is specific for the Sendai virus (10). Relevance of sialylated oligosaccharides in virus infection processes has been also documented with Orthomyxoviruses, such as the Influenza A virus, which recognizes both NeuAc α 2,6Gal- and NeuAc α 2,3Gal-containing receptors (25).

In summary, our results show the relevance of NeuAc α 2,3Gal structures on target cells for the LPMV, because incubation of the virus with this oligosaccharide, as well as in competence assays with a plant lectin specific for this oligosaccharide, abolished the infection induced by this virus. Moreover, our results confirm that Ortho- and Paramyxovirus possess highly preserved similarities in their sugar binding specificity (2).

This work was financed in part by CONACyT (Grants 2151PM and F-643-M9406) and PAPIIT-UNAM (IN209295) Mexico.

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