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Low-pH-induced fusion of Vero cells infected with Junin virus

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Summary. Junin virus (JV) infected Vero cells were used to investigate virus capacity to induce cell–cell fusion. Polykaryocyte formation due to JV was found to be pH and temperature-dependent. A reduced fusion activity was detected on BHK-21 cells. Different JV-strains exhibited a similar extent and pH dependence of their fusion activity. Neutralizing antibodies against the main viral glycoprotein (GP38) inhibited syncytium production and GP38 conformational changes in response to acid treatment were detected by an immunoprecipitation assay.

Introduction

Junin virus (JV), a member of the family *Arenaviridae*, is an enveloped RNA virus with a fragmented helicoidal nucleocapsid. The virion contains two major proteins: a nucleocapsid associated protein NP (MW 60–64 KD), an external glycoprotein GP38 (MW 38 KD, G1) and a minor glycoprotein GP50 (MW 45–50 KD, G2) [19, 22]. GP38, which has been claimed to induce neutralizing antibodies, seems to be the only glycoprotein exposed on the virion surface as it was shown by in vitro iodination of intact purified virions [19].

Enveloped viruses use membrane fusion to deliver their genomes and accesory proteins into the cytosol of host cells. In a previous report we presented evidence that JV enters Vero cells through a receptor mediated endocytic mechanism requiring a low pH-dependent membrane fusion. When the normal route of entry into Vero cells is blocked by a lysosomotropic agent, infection can be induced by fusion at the plasma membrane [4]. Fusion activity of other arenaviruses as lymphocytic choriomeningitis virus (LCMV) and Lassa virus using liposomes was shown to occur at acidic pH [6, 7, 13].

Many families of enveloped viruses exhibit fusion activity in cell cultures. Such activity can be induced directly by virus particles (fusion from without) or after the synthesis of virus-specific products (fusion from within) [28]. In this paper we report that under certain conditions JV produce polykaryons on

infected Vero or BHK-21 cells providing evidence that conformational changes on the major viral glycoprotein occurs at low pH.

Materials and methods

Virus and cells

The following strains of JV were used: IV_{4454} strain obtained from a mild human case, XJC13 strain derivative of the prototype strain XJ [2] and C167, host range mutant obtained by mutagenesis from XJC13 strain [24]. Virus strains were propagated on Vero cells and twice plaque-purified before used. Infectious virus was titered on Vero cells by plaque assay.

Vero and BHK-21 cells were grown in Eagle's minimal essential medium (MEM, Gibco) containing 5% inactivated calf serum and 50 μg/ml gentamycin. Maintenance medium (MM), pH 7.5, consisted of MEM supplemented with 1.5% calf serum and gentamycin.

JV (strain IV_{4454}) was purified from supernatants of infected Vero cells harvested at 48 and 72 h post-infection (p.i.) [19] and purified virus was labelled with ^{125}INa (Conea, Argentina) using IODO-GEN reagent (Pierce).

Syncytium formation assay in JV infected cultures

Vero or BHK-21 cells grown on coverslips, were infected with JV at the indicated multiplicity of infection (moi). At 24 or 48 h p.i. cells were washed with PBS and incubated for different times in MEM containing 0.2% bovine seralbumin (Sigma) and buffered by 10 mM HEPES and 10 mM PIPES. Different amounts of a bicarbonate sodium solution were added to give the desired pH [17]. After that, cultures were washed and incubated for 15 h a 37 °C in MM (pH 7.5), fixed with methanol, stained with Giemsa's (0.4%) and examined for the presence of multinucleated cells [9]. Percentage of fused cells was calculated from 20 randomly selected fields as (number of nuclei within the confines of polykaryons/total number of nuclei in the field) × 100.

To study the effect of anti-JV antibodies on syncytium formation, infected Vero cells were incubated at 23 h p.i. in MM containing several dilutions of anti-JV immunoglobulins (Ig) purified from hyperimmune rabbit serum [21] or anti-JV monoclonal antibodies (mAbs) reactive against GP38 glycoprotein [23]. Cells were incubated 60 min at 37 °C and then cultures were washed, incubated in buffered MEM at pH 5.0 for 2 h at 37 °C and processed as described above. The percentage reduction of syncytium formation by antibodies was calculated with respect to the number of syncytia induced in the control experiment.

Indirect immunofluorescence (IF) assay

Vero or BHK-21 cells grown in coverslips were infected with JV (IV_{4454} strain) at different moi. At 24 h or 48 h p.i. cultures were incubated 2 h at 37 °C or 4 °C and then monolayers were fixed in 4% paraformaldehyde and 50 mM ammonium chloride, incubated with mAb GB03-BE08 [23] and reactive with goat anti-mouse antibody conjugated to fluorescein isothiocyanate. The percentage of fluorescent cells in each preparation was calculated from 20 randomly selected fields of approximately 100 cells each.

Virus neutralization assay

Ig purified from hyperimmune rabbit serum and mAbs were assayed for plaque reduction neutralization as described by Sanchez et al. [23] Neutralization titres are given as $\log_{10}$

values of the dilution causing a 50% reduction in the number of plaque forming units (PFU) in Vero cells compared to control virus.

Immunoprecipitation of viral proteins

Purified radiolabelled virions (IV_{4454} strain) were incubated in PBS at pH 7.1 or 5.0 for 30 min at 37 °C. The acid treatment was stopped by addition of a pre-titrated volume of NaOH 0.1N. Then samples were half diluted with RIPA buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% DOC, 1 mM PMSF) and immunoprecipitated with normal rabbit serum or mAb OB02-BG03 [24]. Immunoprecipitates were resuspended in RIPA buffer and examined for radioactivity in a liquid scintillation counter (Packard). Percentage of immunoprecipitated glycoprotein was calculated as: (immunoprecipitated CPM from virus exposed at different pH/CPM present in each sample before immunoprecipitation) × 100. Nonimmune serum precipitated less than 1000 CPM of ^{125}I-JV pre-treated at both pH, value which was subtracted from the obtained above.

Results

Effect of pH and temperature on polykaryocyte production

To examine the effect of pH on JV membrane fusion, we studied polykaryocyte formation in JV-infected Vero cells that were incubated at 24 h p.i. in medium at different pH values, for 2 h at 37 °C (Fig. 1) (fusion from within). Syncytium production was detected on cultures treated from pH 5.5 downwards, while no fusion was observed neither in infected cells at higher pH values nor in uninfected cells exposed at different tested pH. An increase in both the percentage of fused cells and the number of nuclei per polykaryocyte was observed as the pH was lowered.

No cell fusion was detected when acid treatment was done immediately after viral adsorption to Vero cells (fusion from without) even if variations were made in the moi (1 to 10) and in the period of exposition to acid pH (2 min to 3 h).

The fusion reactions of most enveloped viruses have been found to be temperature-dependent [25]. When we examined syncytium formation in infected Vero cells incubated at pH 5 for 2 h, a temperature dependence of fusion was found (Fig. 1). While a reduction on fusion extent was evident at 25 °C respect to 37 °C, no polykaryocyte was seen in cultures incubated in low pH medium at 4 °C. The percentages of fluorescent cells determined by a cell surface IF assay were 7.6% and 11.5% for cultures incubated for 2 h at 24 h p.i. at 4 °C and 37 °C respectively.

Effect of moi on the rate of cell fusion

Vero cells monolayers were infected at moi 0.1, 0.5 or 1 respectively and 24 h later cultures were incubated in low pH medium. At different times, cells were washed, maintained at pH 7.5 for 15 h and fixed as already described in Materials and methods. As it is shown in Fig. 2, the kinetics of fusion obtained under different moi were preceded by a lag time. The duration of the lag phase decreased with increasing moi. The fusion degree was dependent on the duration of acid treatment and maximal percentage of fused cells was observed in cultures

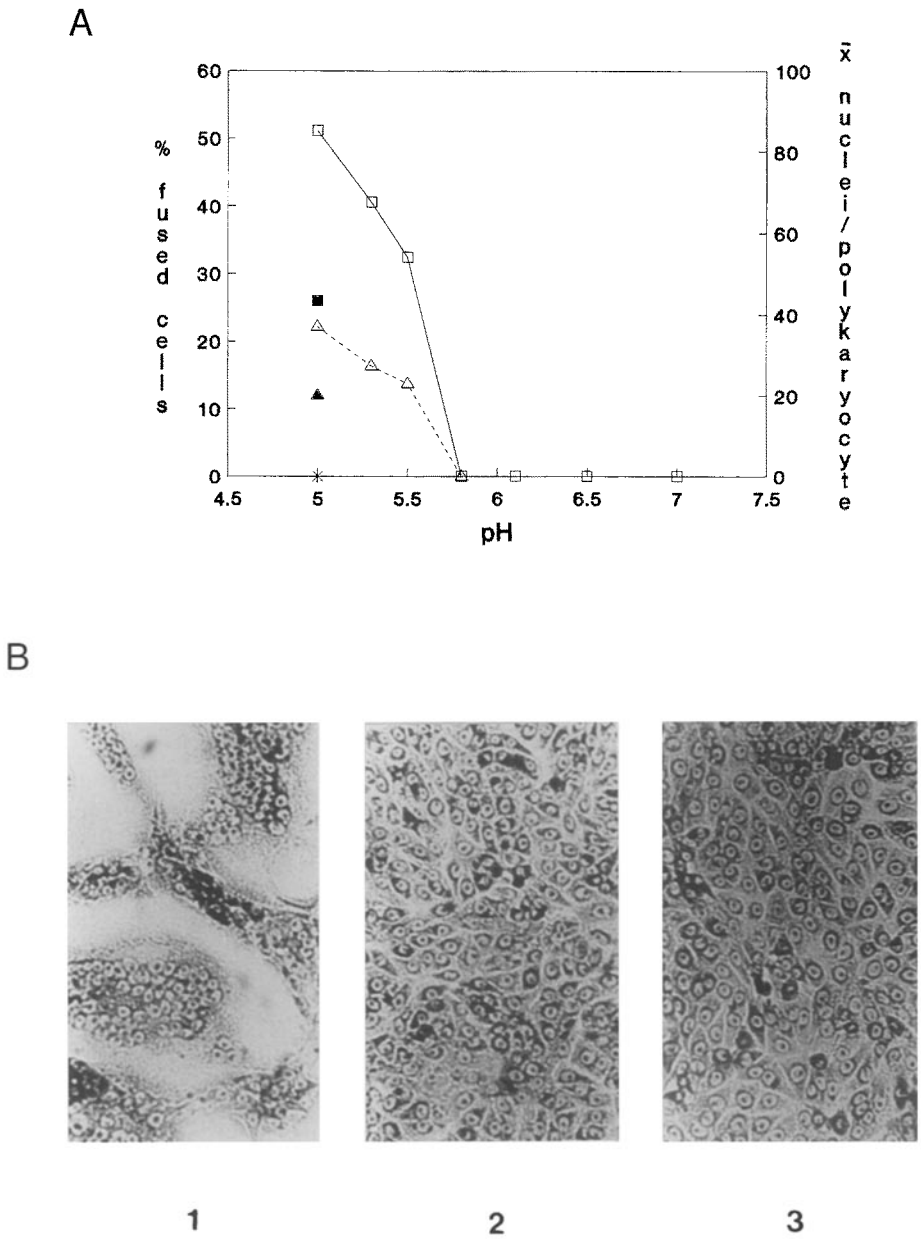

Fig. 1. Effect of pH on syncytium formation. **A** Vero cells were infected with JV (strain IV_{4454}) at moi 1. At 24 h p.i. cultures were incubated with buffered MEM at the stated pH values for 2 h at 37 °C or at pH 5.0 for 2 h at 25 °C or 4 °C. Fixed cultures were stained with Giemsa and examined for multinucleated cells. Results are expressed as percentage of fused cells from cultures incubated at 37 °C (□), 25 °C (■) or 4 °C (*) and number of nuclei per polykaryocyte counted from cultures incubated at 37 °C (△) or 25 °C (▲). **B** JV infected Vero cells were incubated at 24 h p.i. in MEM buffered at pH 5.0 (*1*) or 7.0 (*2*) and stained with Giemsa; *3* uninfected Vero cells. × 250

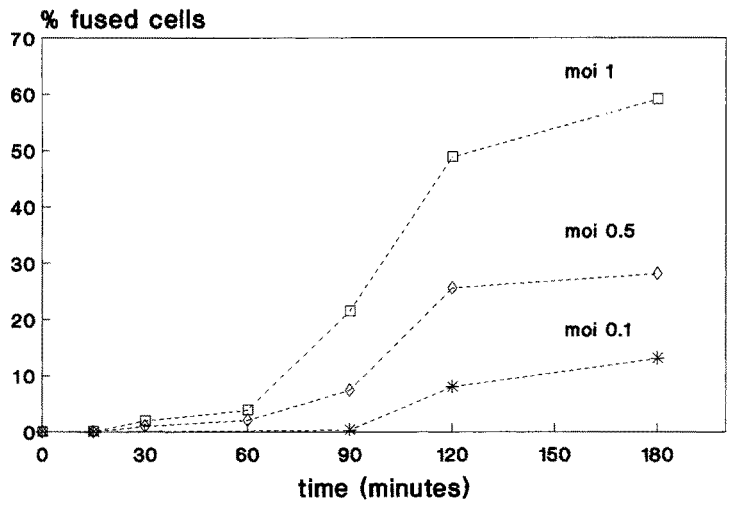

Fig. 2. Effect of moi on the rate of fusion induced at low pH. IV_{4454}-infected cells were exposed for various times to acid medium (pH 5.0) at 24 h p.i.. Then cells were washed, incubated in MM (15 h) and fixed. The extent of fusion was expressed as percentage of fused cells

infected at moi 1 and treated in acid medium for 180 min. A similar moi dependence was observed for GP38 expression at the cell surface. The percentage of fluorescent cells at 24 h p.i. increased from 0.5% at moi 0.1 to 5.2% at moi 0.5 and 11.5% at moi 1.

Influence of cells and virus strain on syncytium formation

For other enveloped viruses (e.g. HIV, influenza and Sendai virus), it is known that viral and host factors contribute to the syncytial phenotype [25, 28, 29]. The influence of host cells on polykaryocyte formation was studied by infecting Vero and BHK-21 cells with IV_{4454} strain (moi 1), as both cellular types produced similar amounts of extracellular virus at 24 h p.i. (data not shown). In contrast to the high percentage of fused cells observed on Vero cells, a reduced fusion activity was detected on BHK-21 cells. In addition low levels of GP38 expression was observed at 24 h and 48 h in BHK-21 cells (Table 1).

A similar threshold of pH for viral fusion activity was observed when syncytium assay was done by infecting Vero cells with different JV strains (Fig. 3). In all cases, no cell fusion was detected in cultures treated with medium at pH values over 5.5. In addition, the increase in fusion activity as the pH was lowered was similar to that previously shown in Fig. 1.

Effect of JV-antibodies on polykaryocyte production

To establish whether the viral envelope proteins are responsible for mediating membrane fusion, syncytium assay was run in the presence of anti-JV immunoglobulins or mAbs reactive against GP38 glycoprotein.

Table 1. Influence of host cell on polykaryocyte production

Hours pi	Percentage of fused cells		Percentage of fluorescent cells	
	Vero	BHK-21	Vero	BHK-21
24	50.0	1.1	11.5	2.2
48	74.0	7.0	55.1	6.8

Vero or BHK-21 cells were infected with IV_{4454} (moi 1). At 24 or 48 h p.i. cultures were incubated in buffered MEM (pH 5.0) for 2 h at 37 °C and examined for syncytium production 15 h later. Fluorescent cell population was expressed as percent of total, at indicated times p.i.

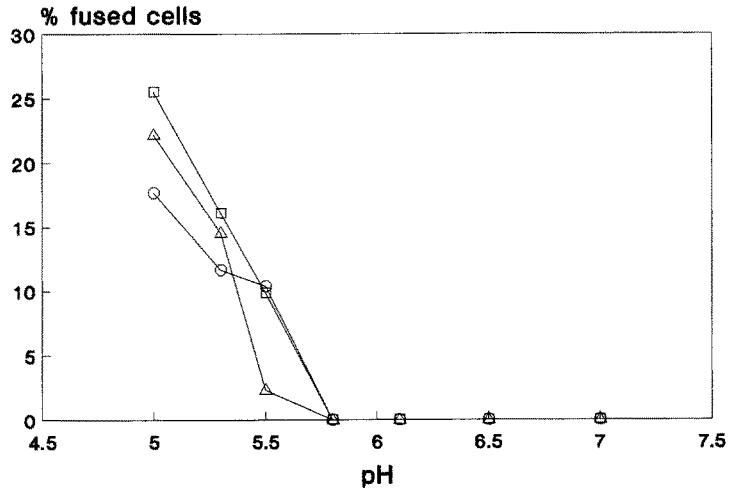

Fig. 3. Influence of JV strains on polykaryocyte production. Vero cells were infected with IV_{4454} (□), XJC13 (△) or C167 (○) strains of JV at a moi of 0.1. At 48 h p.i. cells were incubated 2 h at 37 °C in buffered MEM at different pH values. Then cultures were processed as described in Fig. 1

An important inhibition in the percentage of fused cells and in the number of nuclei per polykaryocyte was obtained when anti-JV immunoglobulins were added 1 h before acidic treatment (Table 2).

Infected cultures incubated with non-neutralizing mAbs exhibited an extent of fusion similar to control experiments. In contrast, both QC03-BF11 and GB03-BE08 neutralizing antibodies produced a strong inhibition of cells fusion and a reduced number of nuclei per polykaryocyte (Table 2).

Table 2. Inhibition of cell fusion by anti-JV antibodies

Treatment	% fused cells	% inhibition	$\bar{x}$ nuclei/ polykaryocyte
control	51.1	–	42
Ig 1:5	5.2	90.0	11
Ig 1:50	17.6	65.5	15
QC03-BF11[a]	<1.0	98.2	9
GB03-BE08[a]	<1.0	99.2	7
QB02-BG03[b]	47.7	6.6	37
EC05-AA04[b]	50.7	0.8	38

Vero cells were infected with JV (strain IV_{4454}) at moi 1 and at 23 h p.i., cultures were incubated in MM in the absence (control) or in the presence of anti-JV Ig (dilution 1:5 or 1:50) or anti-GP38 mAbs: QC03-BF11, QB03-BE08, QB02-BG03 or EC05-AA04 (dilution 1:100), for 1 h at 37 °C. After this treatment cells were exposed 2 h in low pH medium (pH 5.0) and processed as described above

[a] Neutralizing mAbs, neutralization titres were: 3.43 (Ig), 4.85 (GB03-BE08), 4.31 (QC03-BF11)

[b] Non-neutralizing mAbs

Low pH-induced conformational change in GP38

It is known that the acidic environment within endosomes induces conformational changes in viral glycoproteins of many enveloped viruses with pH-dependent fusion activity. These changes, required for virus-host-cell membrane fusion, have been monitored by studying the interaction between radiolabelled or unlabelled viral proteins with monoclonal antibodies [8, 12, 14, 26, 27]. In order to study the effect of low pH on GP38 conformation, radiolabelled virions pre-incubated at pH 5.0 or 7.1 were immunoprecipitated at neutral pH with mAb QB02-BG03. As it is shown in Fig. 4, viral suspensions previously exposed at low pH exhibited a reduction of 75% in GP38 immunoprecipitation with respect to those exposed at neutral pH.

Discussion

Viruses that enter cells through a pH-independent path can form syncytia at neutral pH; those that require a low pH environment for fusion activity such as vesicular stomatitis virus (VSV), Semliki forest virus (SFV) and influenza virus, produce syncytia at low pH (10, 28). When virus proteins were detected on the cell surface (Table 1), JV-infected Vero cells were found to express a fusion function after treatment at acid pH (Fig. 1). Beyond the threshold pH value of 5.5, cell fusion was no longer detectable and within the assayed pH range the fusion extent was maximal at pH 5.0 in cultures incubated at 37 °C. These results confirm the pH dependence of JV mediated syncytium production. In addition,

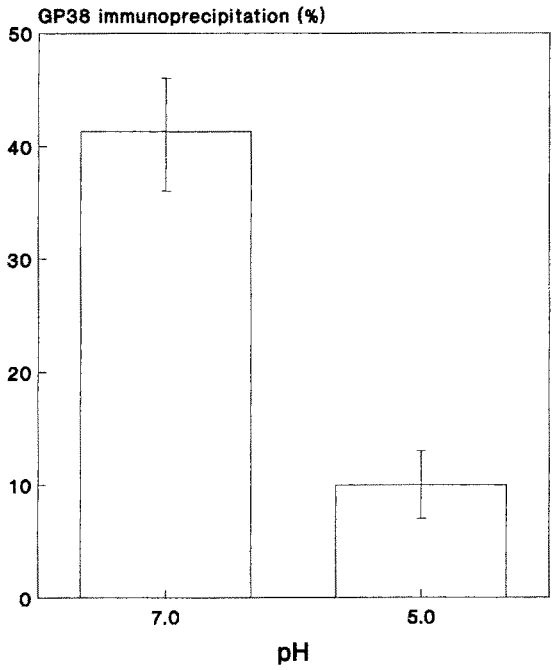

Fig. 4. Immunoprecipitation of ^{125}I-GP38. Iodinated-JV (100.000 CPM) was incubated at pH 7.1 or 5.0 and then immunoprecipitated at neutral pH with mAb OB02-BG03. Values are expressed as percentage of GP38 present in each sample before immunoprecipitation and represent means of 2 experiments ± standard deviation

exposure of JV at 37 °C during 30 min or 2 h a pH 5.0 has no effect on viral infectivity neither in the presence nor in the absence of target membranes (data not shown).

We were not able to demonstrate Vero cells fusion from without mediated by JV. This could be attributed to a very low efficiency of JV adsorption, as we have already described [4], or other parameters of the assay like cell density or virus concentration.

Cell fusion is also temperature dependent: at pH 5.0 polykaryons were detectable only at 37 °C and 25 °C (Fig. 1). The absence of fusion at 4 °C is not due to the lack of GP38 expression at the cell surface. As it has been proposed that integral membrane proteins are key players in fusion reaction [29] the temperature dependent might be attributed to the constraints on the mobility of membrane viral glycoproteins at lower temperatures [15].

Fusion of JV-infected Vero cells increased as a function of time after lowering the pH to 5.0 (Fig. 2). These results suggest that syncytium formation is not a strictly synchronized process [16]. The fact that low periods of 2–3 h at low pH are needed for important cell fusion make it difficult to analyse the kinetics of fusion process. This type of assay might be optimally performed in a different system, like artificial membranes. Further, similar to LCMV [7], JV glycoproteins might be weak fusogenic proteins.

Low levels of GP38 expression on cell surface might be responsible for the reduced fusion activity detected on BHK-21 cells. However, the lipid composi-

tion of the target membrane [25] and other host cell factors [29] could also explain differences observed in polykaryocyte formation between Vero and BHK-21 cells (Table 1). These results differ from those reported by Di Simone et al. [6] who did not detect LCMV-mediated syncytia neither in Vero nor in BHK-21 cells.

The membrane fusion activity of enveloped animal viruses is catalized by the spike glycoproteins. A fusion protein is defined as an integral membrane protein that, upon trigger, changes conformation so as to expose an hydrophobic domain that promotes mixing of lipid components from two bilayers. Recently, a model of cell fusion has proposed that a protein-facilitated pore is a common theme for viral and cellular fusion [18, 29]. We found that neutralizing antibodies reactive against GP38 inhibited syncytium production (Table 2), providing evidence that the external glycoprotein play a role in the fusion process. Though the mechanism of neutralization of infectivity by these antibodies remain to be determined, it is possible that their binding to GP38 prevent viral spike conformational changes, which are usually required for fusion activity. Even if neutralizing antibodies (Table 2) may interfere with the receptor binding it has been shown that viral fusion reactions can take place with membranes that lack receptor [6, 13, 29].

The observed changes in antigenic properties of GP38 that follow the exposure of virions to low pH indicate an acid-induced glycoprotein conformational change (Fig. 4). The sensitivity to proteases provide another method for discriminating between different protein conformations (5, 8). In fact we have also detected an altered sensitivity of GP38 to trypsin digestion after exposure of JV virions to acid pH (data not shown). Recently, conformational changes in the structure of the LCMV-glycoprotein complex following exposure to acidic pH, have been described [7]. In fact, structural reorganization of the viral spike which may represent priming for fusion event has also been reported for SFV, Sindbis, influenza and HIV-1 [1, 11, 20, 26].

Differences in the pathogenesis phenotype for suckling mice among IV_{4454} XJC13 and C167 JV-strains have been associated to changes in GP38 peptide pattern obtained after limited proteolysis [3, 24]. However, Vero cells infected with these strains did not exhibit important differences in the extent and pH dependence of their fusion activity (Fig. 3). So JV-fusogenic activity in Vero cells does not seem to be related to virulence.

Biochemical studies done with different solubilization agents (Castilla, data not shown) suggest that GP38 is an hydrophilic protein, so acid pH-induced conformational changes in JV external glycoprotein may expose some hidden hydrophobic sequence or in turn change the interaction with the other membrane glycoprotein (GP50) exposing a fusion peptide. A previous study on the arenavirus Lassa described the acid-induced fusion activity of a synthetic amphiphilic peptide homologous to an aminoacid sequence localized in the internal glycoprotein G2 [13]. Further studies need to be done in order to determine the role of the internal viral glycoprotein in JV-membrane fusion activity.

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