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The entry of Junin virus into Vero cells

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Summary. The entry mechanism of Junin virus (JV) into Vero cells was studied analyzing the effect of lysosomotropic compounds and acid pH on JV infection. Ammonium chloride, amantadine, chlorpheniramine and procaine inhibited JV production. The action of ammonium chloride was exerted at early times of infection. Virus internalization was inhibited and viral protein expression was not detected. When the extracellular medium was buffered at low pH, the ammonium chloride induced block on JV infection was overcome. Furthermore, JV was able to induce fusion of infected cells at pH 5.5 leading to polykaryoctye formation. Taken together, these results demonstrate that JV entry occurs through an endocytic mechanism requiring a low pH dependent membrane fusion.

Introduction

Junin virus (JV) is a member of the *Arenaviridae* family of enveloped RNA viruses. Virions contain a genome composed of two segments of single-stranded RNA and two major structural proteins, the nucleocapsid protein (NP, MW 60–64 kDa) and an external glycoprotein (GP38, MW 38 kDa) [20]. Other minor proteins have also been described in purified virions or in infected cells [5, 6, 10].

The main biological properties of JV are its ability to establish persistently infected cultures in-vitro and to produce chronic infections in the field mouse *Calomys musculinus* [27]. In humans, JV induces Argentine haemorrhagic fever, an endemoepidemic disease with hematologic and neurologic signs, which mainly affects male rural workers [27].

Although some features of arenavirus replication have been described, the early events of the multiplication cycle remain poorly characterized. Main interest has been focussed on the knowledge of viral RNA transcription and replication, the characterization of the ambisense genome strategy and the study of protein structure and expression [2, 3]. Little is known about the process

of virus attachment and entry into the cell. A brief report has recently suggested that the replication of the arenaviruses Pichinde, Mopeia and Lassa was sensitive to some lysosomotropic compounds [8].

Two distinct pathways operate for the entry of enveloped viruses into animal cells: some viruses penetrate by direct fusion of the viral envelope with the plasma membrane while other viruses are taken up by an endocytic mechanism [15]. The endocytosed virions travel to intracellular compartments where acidic conditions facilitate fusion between the viral envelope and the vesicle membrane releasing the nucleocapsid into the cytoplasm. Thus, lysosomotropic agents such as weak bases and carboxylic ionophores that elevate the pH of acidic organelles, interfere with the virion uncoating and inhibit the replication [1, 9, 14]. The uncharged form of any weak base has been proposed to enter the acidic intracellular compartments very efficiently, raising the pH as it becomes protonated and inhibiting hydrolytic enzymes [15].

In this paper, we report studies on JV penetration into Vero cells analyzing the effect of acidic pH and lysosomotropic bases on virus entry.

Materials and methods

Cells and virus

Monolayers of Vero cells were grown in Minimum Essential Medium (MEM) supplemented with 5% calf serum and gentamycin. The IV4454 strain of JV [4] was used.

Lysosomotropic compounds

Stock solutions of amantadine (SPECIA), ammonium chloride (Fisher Company), chlorpheniramine (Schering-Plough) and procaine (Schering-Plough) at concentrations of 5, 500, 100 and 50 mM, respectively, were prepared in culture medium and sterilized by filtration.

Effect of time of addition of ammonium chloride on JV replication

Ammonium chloride (15 mM) was added to Vero cells 1 h before infection with JV or at 0, 1, 3, 5 or 8 h post-infection (p.i.). In all cases supernatant cultures were harvested at 24 h p.i. and extracellular virus yields were determined by plaque assay.

Adsorption assay

Vero cells were infected with JV (virus inoculum: 2×10^5 PFU) in the presence or in the absence of 15 mM ammonium chloride. After adsorption for 0, 10, 20, 30, and 60 min at 4 °C, infected cells were washed twice with cold phosphate buffer saline (PBS) and disrupted by freezing and thawing. The amount of infectious bound virus was then determined by plaque assay.

Internalization assay

Cultures of Vero cells were infected with JV at a moi of 1. After 30 min adsorption at 4 °C two washes were done to remove excess inoculum and then cultures were warmed to 37 °C for various times in the presence or absence of 15 mM ammonium chloride. Cultures were

subsequently treated with proteinase K (0.25 mg/ml) in PBS for 30 min at 4 °C in order to remove external virus. Protease treatment was stopped by the addition of 2 mM phenyl methyl sulfonyl fluoride (PMSF), 3% bovine serum albumin (BSA) in PBS. Cells were centrifuged 10 min at 2 000 **g** and the resultant pellet was washed and resuspended in MEM. Internalized virus was measured by an infectious center assay on Vero cells.

Indirect immunofluorescence assay

Vero cells grown in coverslips were infected with JV (moi 1) and 15 mM ammonium chloride was added to the culture medium after adsorption. At 24 h p.i. supernatants were removed. Monolayers were washed with cold PBS, fixed in methanol and stained for cytoplasmic immunofluorescence with anti-JV purified immunoglobulins as previously described [23].

pH dependence of JV internalization

Vero cell monolayers in 24-well plates were adsorbed for 1 h at 4 °C with JV in MEM containing 0.2% BSA, 15 mM HEPES, pH 7.5. In a set of cultures cells were washed twice with PBS and then warmed to 37 °C by the addition of pre-warmed medium buffered with 10 mM HEPES and 10 mM 1–4-piperazinediethene-sulfonic acid (PIPES) at pH 5.5, 5.8, 6.1, 6.3, 6.6 and 7.5, in the presence or in the absence of 15 mM ammonium chloride. After 3 h of incubation at 37 °C, cells were washed twice with PBS and MEM containing 0.7% methylcellulose, pH 7.5, was added. In other set of cultures, after adsorption cells were washed with PBS and incubated at 37 °C for 2 min in MEM buffered at different pH as indicated above. Then, cells were washed with PBS and MEM at pH 7.5 containing or not 15 mM ammonium chloride was added. After 3 h of incubation cultures were overlaid with methylcellulose as above. In both set of cultures plaques were counted after 7 days of incubation and percent of inhibition was calculated.

Effect of pH on JV induction of syncytia in Vero cells

Vero cells were infected with JV at a moi of 1. At 48 h after infection, medium was removed and cells were incubated in MEM buffered at pH 5.5 or 7.5 as indicated above. Then, monolayers were stained with Giemsa and syncytia containing more than 10 nuclei were counted.

Results

Inhibition of JV infection by lysosomotropic agents

The effect of several lysosomotropic drugs (procaine, chlorpheniramine, amantadine and ammonium chloride) on virus production was first examined. Previously the lack of toxicity of the four bases for Vero cells was investigated by assessing their effects on cell growth (Fig. 1). The highest noncytotoxic concentrations were 20 mM for ammonium chloride, 0.6 mM for amantadine and chlorpheniramine, and 3 mM for procaine. As seen in Fig. 1 JV infection was inhibited by the drugs in a dose-dependent manner. The most active compound in the range of noncytotoxic concentrations assayed was ammonium chloride which reduced JV yield more than 3 log units, thus it was chosen for the next experiments. The presence of the compounds at the concentrations used did not affect either the pH of the culture medium or the infectivity of JV particles (data not shown).

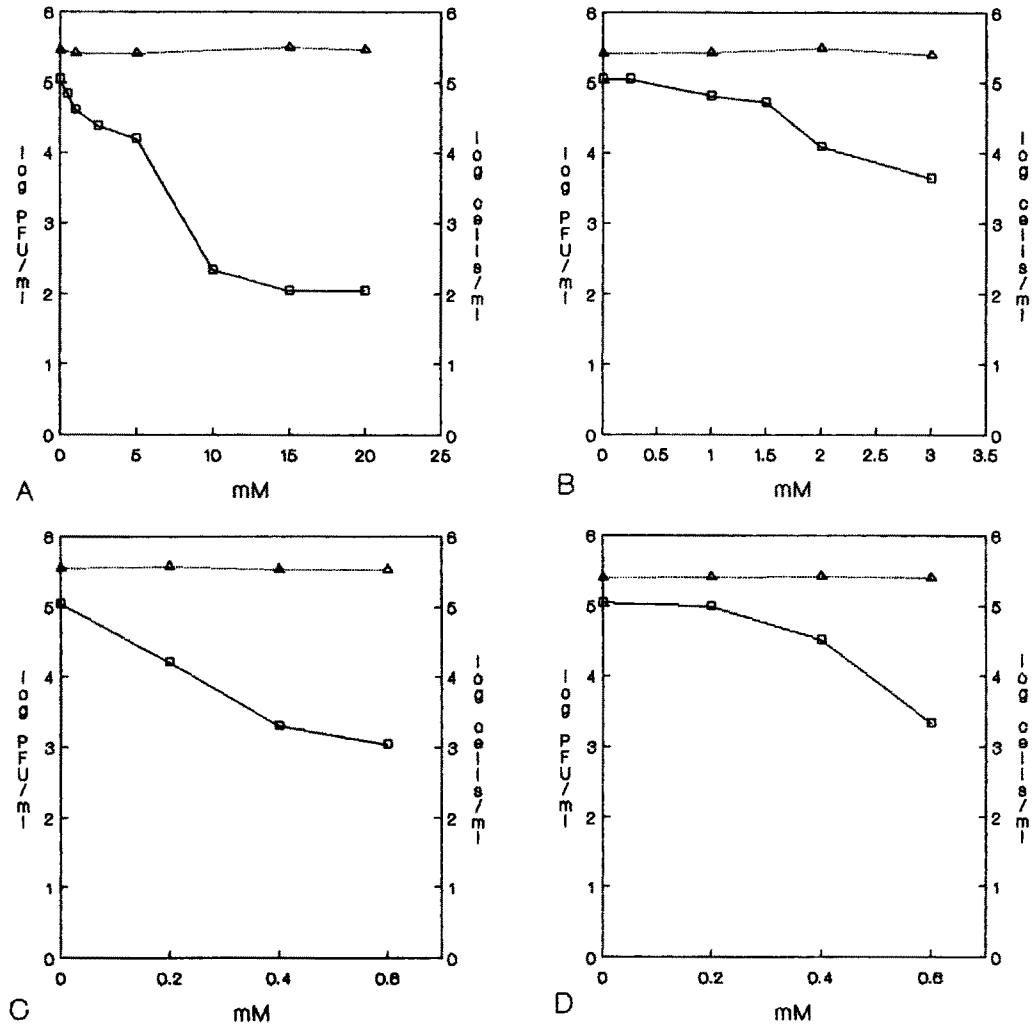

Fig. 1. Dose-dependent inhibition of JV infection by lysosomotropic compounds. Vero cell monolayers were incubated in the presence of different concentrations of ammonium chloride (**A**), procaine (**B**), amantadine (**C**), and chlorpheniramine (**D**). After 24 h of incubation at 37 °C the number of viable cells was determined by the Trypan blue exclusion method (——△——). Other set of cultures were infected with JV (moi 1) in the presence of noncytotoxic concentrations of the compounds. The drugs were maintained for 24 h, supernatants were then harvested and viral titers were determined (——□——)

Effect of the time of addition of ammonium chloride on JV replication

To determine the time at which lysosomotropic agents caused their inhibitory action, we next examined the effect of the time of addition of 15 mM ammonium chloride on extracellular virus yields. A nearly complete inhibition of viral multiplication was observed when the compound was added one hour before or simultaneously with virus infection and maintained during all the period of incubation (Fig. 2). When time of drug addition was delayed, virus production progressively increased, indicating that ammonium chloride inhibited an early

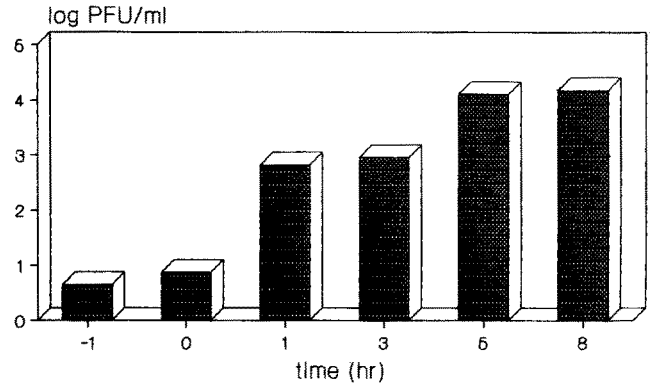

Fig. 2. Effect of the time of drug addition of ammonium chloride on JV replication. Ammonium chloride was added to Vero cells at different times of JV infection. Time 0 was defined as the time when virus was added to start the infection. Extracellular virus yields were determined at 24 h p.i. Virus titer in control culture without compound treatment: 1.4×10^5 PFU/ml

step of the replicative cycle. In fact, no significant differences were found when the drug was added between 5 and 8 h p.i.

Effect of ammonium chloride on JV adsorption, internalization and protein expression

Since maximum inhibition in JV multiplication was obtained when the drug was present during the first hour after infection, we examined its effect on the early stages of replication. As shown in Table 1, ammonium chloride had no effect on JV adsorption since the titers of virus adsorbed at 4 °C in treated and untreated cells did not differ significantly. In both cases, virus adsorption occurred rapidly and the percentage of adsorbed virus was approximately 5%.

Table 1. Effect of ammonium chloride on JV adsorption

Time post-adsorption (min)	Adsorbed virus (log PFU/ml) ammonium chloride (mM)	
	0	15
0	3.98	3.97
10	4.05	3.90
20	4.26	4.17
30	4.30	4.40
60	4.50	4.50

JV was adsorbed to Vero cells at 4 °C for different periods of time, in the presence (15 mM) or absence (0 mM) of ammonium chloride, and the cell-adsorbed virus was determined

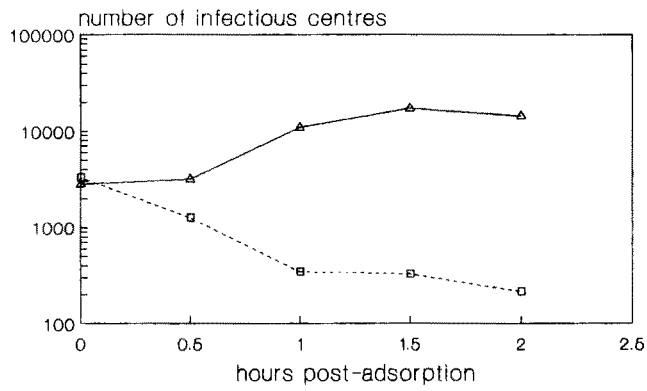

Fig. 3. Effect of ammonium chloride on JV internalization. Cultures of JV-adsorbed Vero cells were warmed to 37 °C for 0.5, 1, 1.5, and 2 h post-adsorption in the presence (--□--) or in the absence (——△——) of 15 mM ammonium chloride. Internalized virus was quantitated by infectious center assay as described in Materials and methods

Similar results were obtained when non-adsorbed virus was assayed (data not shown).

To investigate whether ammonium chloride affects JV internalization, virus adsorbed cells at 4 °C were warmed to 37 °C for various intervals in presence or absence of the compound and internalized virus was determined by infectious center assay. The amount of JV internalized virus in untreated cells sharply increased after 30 min of incubation at 37 °C and the uptake was apparently complete after 1.5 h (Fig. 3). In the presence of ammonium chloride there was no virus internalization and a progressive decrease is observed in the number of infectious centers. The relatively small difference between the control and treated cells at the 30 min time point might reflect the fact that part of the adsorbed virus was not removed by the proteinase K treatment.

The action of weak bases on JV replication was confirmed by measuring viral protein production in infected cells by an immunofluorescence assay. The inhibition in the number of fluorescent cells in the presence of 15 mM ammonium chloride was 98.8% (Fig. 4). The other lysosomotropic compounds also inhibited viral antigen expression (data not shown).

pH dependence of JV internalization

In an effort to demonstrate that the entry of JV into Vero cells is an acid pH-dependent process, we have finally studied the effect of different pH values on the internalization of JV particles that have been prebound to Vero cells. In the absence of ammonium chloride, infectivity of JV was not affected at the pH range assayed (5.5–7.5) (data not shown). A high degree of inhibition was observed when cultures were incubated in medium containing ammonium chloride at pH 7.5, 6.6 and 6.3 during 3 h, while there was only 31.5 and 22.3% inhibition at pH 6.1 and 5.8, respectively (Fig. 5). At more acidic pH values the action of ammonium chloride was almost totally abolished. To assess that

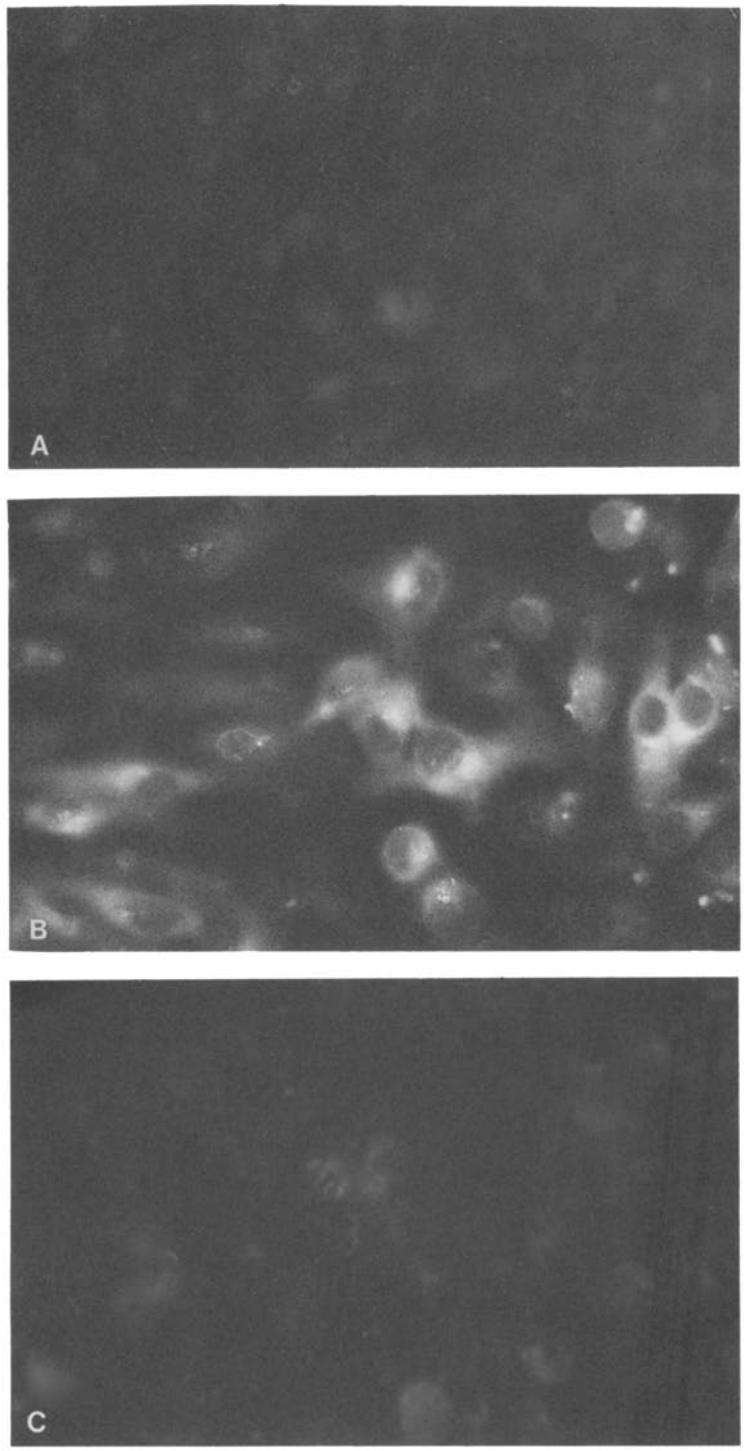

Fig. 4. Action of ammonium chloride on expression of JV proteins. Vero cells were mock infected (**A**) or infected with JV in the absence (**B**) or in the presence (**C**) of 15 mM ammonium chloride. After 24 h of infection indirect immunofluorescence staining was carried out. Magnification: × 400

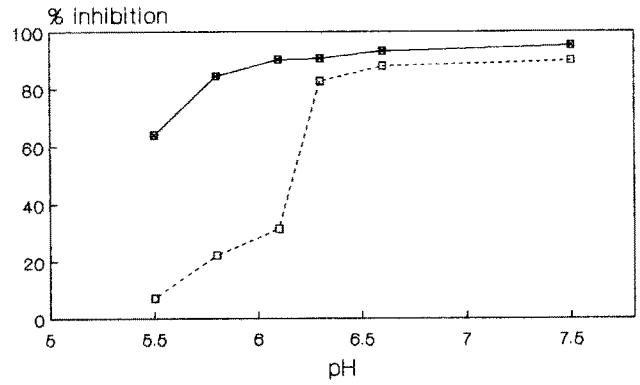

Fig. 5. pH dependence of JV internalization. JV was adsorbed to Vero cells at pH 7.5. A set of cultures (---□---) were then incubated for 3 h in medium buffered at different pH values, in the presence or in the absence of ammonium chloride. Then cells were washed and overlaid with MEM containing methylcellulose, pH 7.5. In other set of cultures (——□——), after adsorption cells were treated for 2 min in MEM buffered at different pH, then monolayers were washed with PBS and incubated for 3 h in MEM at pH 7.5 containing or not 15 mM ammonium chloride. Cultures were overlaid with methylcellulose as above. In both set of cultures plaques were counted and percent of inhibition was calculated as [1 – (number of plaques in ammonium chloride treated cells/ number of plaques in untreated cells)] × 100

the acid pH is modifying the mechanism of viral entry and is not just neutralizing the ammonium chloride, JV bound cells were briefly treated with medium buffered at different pH for 2 min and then incubated for 3 h in neutral medium containing or not ammonium chloride. This treatment prevents endocytosis by inhibiting release into the cytoplasm of virus entered in endocytic vacuoles and allows virus entry only by fusion at the plasma membrane [26]. Under these experimental conditions, ammonium chloride inhibition was partially reversed at pH 5.5 (Fig. 5).

JV-induced membrane fusion at low pH

To reinforce that the membrane fusion activity of Junin virus is expressed at low pH, the formation of JV-induced syncytia in infected Vero cells incubated in medium at pH 5.5 or 7.5 was quantitated. Cell fusion was observed at 48 h p.i. but only at pH 5.5, there being more than 10 nuclei in the syncytia whereas no polykaryon formation was seen at neutral pH (Fig. 6).

Discussion

These studies present evidence that Junin virus entry occurs via a pH dependent endocytic mechanism mediated by a glycoprotein fusogenic activity.

Acidotropic bases are known to disrupt endosomal functions when their uncharged forms enter endosomes and lysosomes, become protonated, raise the pH and inhibit the hydrolytic enzymes [18]. We have demonstrated here

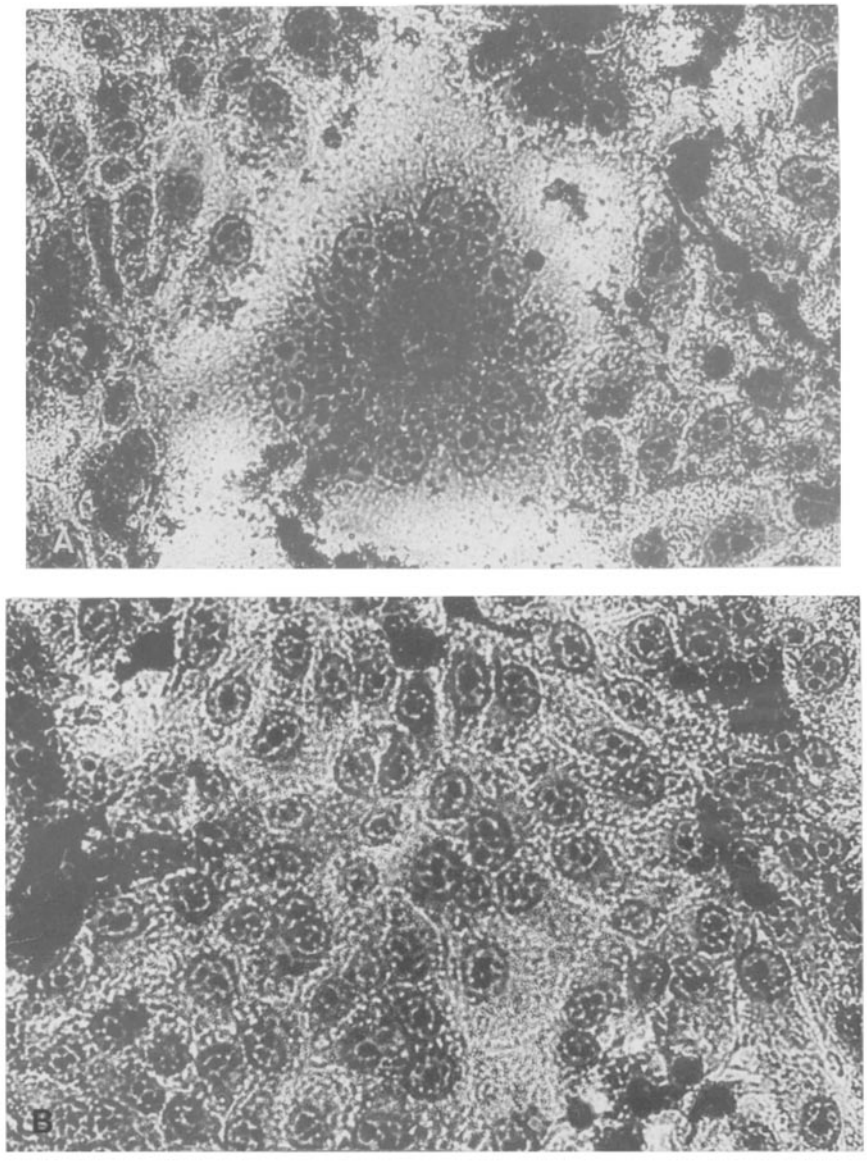

Fig. 6. JV-induced fusion at low pH. Infected Vero cells were incubated at 48 h p.i. in MEM buffered at pH 5.5 (**A**) or 7.5 (**B**) and stained with Giemsa. Magnification: × 400

that the production of JV infectious particles and the expression of viral proteins in infected cells was inhibited by this kind of compounds (Figs. 1, 4).

The main action of ammonium chloride on JV replication was exerted at an early step in the multiplication cycle (Fig. 2). However, virus attachment which occurred at 4 °C was not inhibited as it is shown in Table 1. Virions bound to cells at 4 °C are compelled to internalize when the temperature of incubation is raised to 37 °C. When JV internalization, the next stage of the viral cycle, was studied a significant inhibition was observed during the first

hour after adsorption. In the presence of ammonium chloride an increasing reduction in the number of proteinase K-resistant infectious centers was detected (Fig. 3.) It might be due to the lysosomal degradation of endocytosed virions unable to fuse with endosomal membranes, as seen for other enveloped viruses.

These data were indicative of a process of low pH-dependent endosomal fusion for JV uptake. In fact, a bypass of the ammonium chloride block of JV infection was achieved when the extracellular medium was at a pH below 6.1 (Fig. 5), suggesting that the acidic conditions would probably trigger direct fusion of virus envelope with the cell membrane. The location of penetration is determined by the pH threshold of fusion activity [15]. For some viruses such as Semliki Forest virus, fusion occurs at a pH 6.2 in early endosomes, whereas Influenza virus X-31 requires pH 5.3 and fuses in the late endosomes [12, 24, 25]. For JV entry, fusion seems to occur in endosomes where the pH is 6.1 or lower. The acid environment may be responsible for structural changes in JV external proteins allowing membrane fusion and facilitating viral uncoating as described for Influenza virus [7], Rubella virus [17], West Nile virus [13] and tick-borne encephalitis virus [11].

It is possible to induce fusion in some model systems by mimicking the low pH intracellular conditions. In particular, Mann et al. [16] have shown that Sindbis virus infected cells express a fusion function after treatment at acid pH. We demonstrated that Junin virus can mediate cell fusion at pH 5.5 producing polykaryocytes in which over 40% of the cells in the monolayer participate (Fig. 6). Thus, this result offers indirect support for the conclusion that the route of JV entry is pH-dependent in Vero cells. Major expression of GP38, the main external envelope glycoprotein [19], was observed in the surface of JV infected cells at 48 h p.i., by immunofluorescence assay (data not shown). Thus, GP38 might be responsible for JV-induced membrane fusion in the endosome or in the cell surface. This proposal is also supported by results obtained with C167, a host-range mutant of JV, with an alteration in GP38 detected by peptide mapping and a blockade in adsorption-penetration pathway [21, 22].

In conclusion, our data demonstrate for the first time that JV enters the cell by a receptor mediated endocytic pathway and that low pH is neccesary for viral internalization through a fusing activity. Further experiments are in progress to determine the precise role of GP38 in virus entry and the nature of the conformational changes at low pH leading to membrane fusion.

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