EFFECT OF AMMONIUM CHLORIDE ON MULTIPLICATION OF RINDERPEST VIRUS IN VERO CELLS

D. HEMADRI, S.K. BANDYOPADHYAY*

Division of Virology, Indian Veterinary Research Institute, Mukteswar-Kumaon 263138, India

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Summary. – Amonium chloride, a lysosomotropic weak base, inhibited replication of rinderpest virus in Vero cells. The inhibition of replication was dose-dependent and the minimum effective dose of ammonium chloride was determined as 5 – 10 mmol/l. The fusion efficiency and the yield of both cell-free and cell-associated virus were reduced in the presence of the inhibitor. Western blot analysis of rinderpest virus-infected Vero cells revealed that synthesis of two virus-induced polypeptides were affected by the presence of ammonium chloride.

Key words: rinderpest virus; ammonium chloride; Vero cells; antiviral effect

Introduction

Rinderpest virus is an acute contagious disease of cattle and buffaloes. The disease is caused by a virus of the Morbillivirus genus of *Paramyxoviridae* family and is characterized by high fever, necrosis and erosion of the gastrointestinal tract leading to diarrhoea, dehydration and death (Plowright, 1968). Attenuated virus vaccines, e.g. GTV (Edwards, 1928), lapinized rinderpest virus (Nakamura *et al.*, 1938) and tissue culture rinderpest virus (Plowright and Ferris, 1962) are available against the disease. Rinderpest virus induces one non-structural and seven structural polypeptides in infected cells of which two, F and H are glycosylated (Grubmann *et al.*, 1988).

Ammonium chloride (NH_4Cl), a lysosomotropic weak base, inhibits replication of several viruses either by inhibiting the entry process due to elevated pH in endosomes or by interfering with the glycosylation of viral polypeptides due to pH change in the Golgi complex (Jensen and Liu, 1961; Koyama and Uchida, 1989; Marsh *et al.*, 1981; Matlin, 1986). We report in this paper that NH_4Cl inhibits replication of rinderpest virus *in vitro* at late stage of virus replication.

*Corresponding author

Materials and Methods

Cells. All the work was carried out in Vero cells (ATCC, USA) at the passage level between 125 to 150. The cells were grown in Eagle's Minimum Essential Medium (MEM) (Sigma) containing 5% foetal bovine serum (FBS) or new-born calf serum (NBCS).

Viruses. Tissue culture rinderpest virus (RBOK strain; Plowright and Ferris, 1962) at its 102nd passage in primary calf kidney cells was adapted to grow by three serial passages in Vero cells (vRBOK).

Lapinized rinderpest virus (Nakamura III strain) at its 991st passage in rabbits was adapted to grow in Vero cells by the technique of fusion as described by Ishii *et al.* (1986). Briefly, 2×10^8 splenocytes from a rabbit infected with lapinized virus were fused with 5×10^6 trypsinized healthy Vero cells in 1 ml 42.5% polyethylene glycol 1 500 (Sigma). The fused cells were distributed in several 25 cm^2 and 75 cm^2 flasks in MEM with 10% FBS. Monolayers of fused cells were co-cultivated with fresh Vero cells for two blind passages before rinderpest virus-specific CPE appeared (vLAV).

Determination of minimum effective dose (ED_{50}) of ammonium chloride. Various concentrations of ammonium chloride of tissue culture grade (Sigma) were added one hour after adsorption of the virus to sets of confluent monolayers of Vero cells maintained in MEM containing 2% NBCS. The ED_{50} was determined from the lowest concentration of the inhibitor which inhibited CPE at least in 50% of cells as compared to the control on day 6 p.i. Titration of each virus sample was carried out by the method of Mirchamsy *et al.* (1970).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. SDS-PAGE was carried out according to Laemmli (1970). Separated polypeptides were transferred to nitrocellulose membrane with the help of a blotting apparatus (Atto Corp., Japan). The blot was soaked in 5% skimmed milk powder in sterile PBS for half an hour before incubating with 1:100 dilution of Vero cell-adsorbed rinderpest hyperimmune serum raised in rabbits. Next, the blot was incubated in 1:1000 dilution of pig anti-rabbit horseradish peroxidase conjugate (Dakopatts). The blot was developed using 3,3' di-amino-benzidine (Sigma) as a substrate.

Results

Determination of ED_{50} of ammonium chloride

Confluent monolayers of Vero cells were infected or mock-infected with either vRBOK or vLAV at a multiplicity of infection of 0.01 $TCID_{50}$/cell in the presence of 0,5,10 and 1 mmol/l ammonium chloride. Concentrations higher than 15 mmol/l were toxic to Vero cells (data not shown). Cells were observed daily for appearance of CPE and the results after 6 days of infection are shown in Table 1. Virus infectivity titers of the culture fluids was also determined (Table 2).

Table 1. Inhibition of rinderpest virus-induced CPE in Vero cells by ammonium chloride and determination of ED_{50}

Virus	CPE at NH_4Cl concentration (mmol/l)				ED_{50} (mmol/l)
	0	5	10	15	
vLAV	++++	+++	+	+	10
vRBOK	++++	+	+	+	5

(+), (++), (+++), (++++): the increasing extent of CPE.

Table 2. Effect of ammonium chloride on the yield of rinderpest virus in cells

Virus	Yield (log $TCID_{50}$/ml) at NH_4Cl concentration (mmol/l)			
	0	5	10	15
vLAV	6.3	5.6	4.3	3.3
vRBOK	4.6	3.0	2.3	0.75

The ED_{50} of ammonium chloride was determined as 5 mmol/l for vRBOK and 10 mmol/l for vLAV. The reduction of the infectivity was proportional to the increased concentration of the inhibitor. However, even at 15 mmol/l concentration a significant titer of infective virus could be detected, especially with vLAV.

Effect of ammonium chloride on virus adsorption

In this experiment, confluent monolayers of Vero cells were maintained in MEM containing 15 mmol/l ammonium chloride. Cells were infected with 0.01 $TCID_{50}$ of vRBOK per cell in the presence of the inhibitor which was promptly withdrawn at the end of the 1 hr adsorption period. Total yield of virus on day 5 p.i. was $10^{4.25}$ $TCID_{50}$/ml, while that in the cells which were not exposed to ammonium chloride was $10^{4.6}$ $TCID_{50}$/ml. This clearly demonstrates that ammonium chloride did not affect the entry of rinderpest virus into Vero cells.

Effect of ammonium chloride on virus fusion

Fusion or syncytia formation was one major characteristic of lapinized virus adapted to Vero cell. When confluent monolayers of cells on cover-slips were infected with vLAV in the presence of ammonium chloride, fusion activity of the virus was greatly affected (Fig. 1). Not a single syncytia was detected in the infected monolayer in the presence of 15 mmol/l inhibitor.

Effect of ammonium chloride on virus yield

The yield of cell-associated and cell-free virus in the presence of different concentrations of ammonium chloride was determined in vLAV-infected Vero cells on day 5 p.i. (Table 3). The yield of cell-free virus was always marginally higher than that of cell-associated virus, irrespective of the presence of the inhibitor. However, both were proportionally reduced with the increasing concentrations of ammonium chloride.

Effect of ammonium chloride on polypeptide synthesis in infected cells

Vero cells were infected or mock-infected with vRBOK or vLAV in the presence or absence of 15 mmol/l ammonium chloride. Infected cells were harvested at 60 hrs p.i. and the cytosol fraction was extracted with 0.2 mol/l Tris-HCl containing 1% Nonidet P-40 and 1% PMSF as protease inhibitors. These extracts were subjected to Western blot analysis (Fig. 2 and 3).

Ammonium chloride had detrimental effect on the synthesis of viral polypeptides. The effect of the inhibitor was more severe on cells infected with vLAV (Fig. 2), where two virus-induced immunogenic polypeptides with esti-

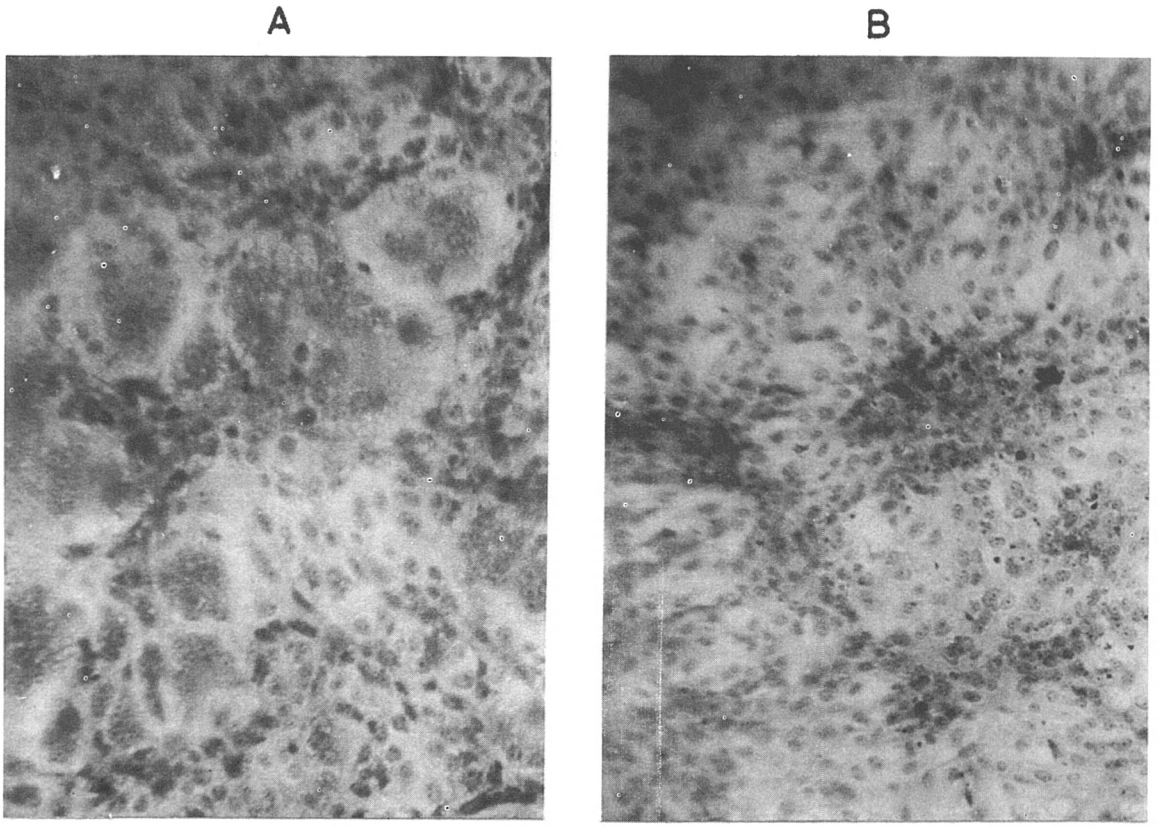

Fig. 1
Effect of ammonium chloride on fusion efficiency of vLAV in Vero cells
A: Virus-infected cells in the absence of the inhibitor 5 days p.i. Note the multinucleated giant cells (syncytia). B: The same as above in the presence of 15 mmol/l ammonium chloride.

Table 3. Yield of cell-free and cell-associated rinderpest virus (strain vLAV) on day 5 p.i. in the presence of various concentrations of ammonium chloride

NH_4Cl concentration (mmol/l)	Yield (log $TCID_{50}$/ml)	
	cell-free virus	cell-associated virus
0	5.75	5.25
5	4.75	4.5
10	3.75	3.3
15	3.3	2.5

mated molecular weights 77 K and 49.5 K were absent and a new polypeptide (44 K) appeared. Although the effect of the inhibitor was less marked on vRBOK-infected cells, the synthesis of the above mentioned two polypeptides were affected too (Fig. 3).

Discussion

The minimum effective dose of ammonium chloride for vLAV and vRBOK viruses was determined in the present study as 10 mmol/l and 5 mmol/l, respectively. Both the concentrations were higher than ED_{50} reported for influenza virus (Jensen and Liu, 1961). However, the sensitivity to ammonium chloride may differ between different viruses as it has been shown here between two attenuated vaccine strains of rinderpest virus. While experimenting with HSV-1, Kousoulas *et al.* (1983) observed that the non-syncytial strains were more sensitive to ammonium chloride as compared to syncytial strains, and lower concentration of the inhibitor had little effect on a particular strain of HSV-1. We also observed that vLAV, whose nature is more syncytial, required higher concentration of the inhibitor as compared to vRBOK. The experiment designed to determine the stage of rinderpest virus replication at which the inhibitor acts, clearly demonstrated that adsorption of the virus to cells was not affected. Marginal decrease in the

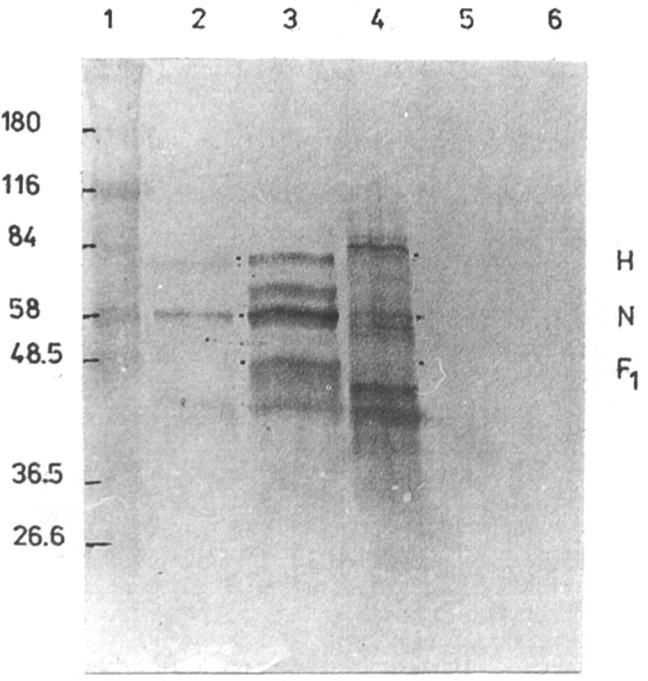

Fig. 2
Immunoblot analysis of the effect of ammonium chloride on the synthesis of immunogenic polypeptides in vLAV-infected Vero cells
Molecular weight markers (lane 1) with relative values (K) on the left side. Purified vLAV (lane 2). vLAV-infected cell lysate 60 hrs p.i. in the absence of the inhibitor (lane 3) and in the presence of 15 mmol/l ammonium chloride (lane 4). Mock-infected cell lysate 60 hrs p.i. in the absence of inhibitor (lane 5) and in the presence of 15 mmol/l ammonium chloride (lane 6). Three major immunogenic polypeptides putatively designated as H,N and F_1 are indicated with dots and letters (on the right side).

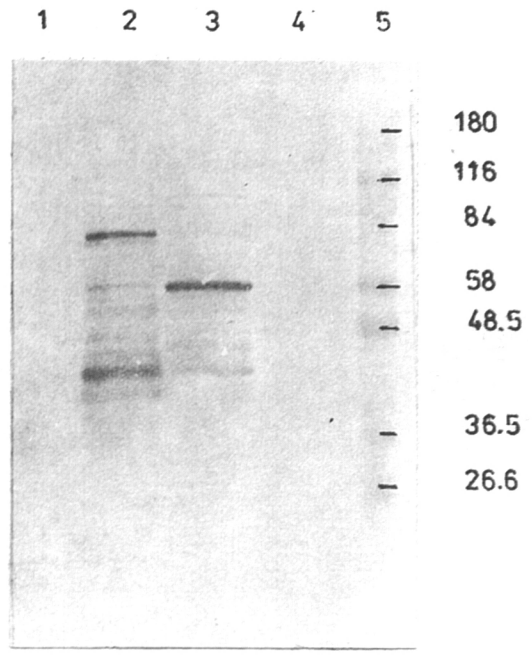

Fig. 3
Immunoblot analysis of the effect of ammonium chloride on the synthesis of immunogenic polypeptides in vRBOK-infected Vero cells
Mock-infected (lane 1) and virus-infected (lane 2) cell lysate 60 hrs p.i. in the absence of the inhibitor. Virus-infected (lane 3) and mock-infected (lane 4) cell lysate in the presence of 15 mmol/l ammonium chloride. Molecular weight markers (lane 5) with relative values (K) on the right side. Putative polypeptides H,N and F_1 indicated on the left side.

yield of the virus could be attributed to the effect of the residual inhibitor following its withdrawal after adsorption process. For HSV-1 and influenza viruses too, it has been reported that ammonium chloride does not interfere at the time of adsorption but possibly at later stages of replication (Koyama and Uchida, 1989; Jensen and Liu, 1961).

The fusion efficiency of rinderpest virus, particularly of vLAV, was drastically inhibited in the presence of ammonium chloride. The fusion or syncytia formation in paramyxovirus-infected cells has been attributed to viral glycoprotein F. Further we observed that ammonium chloride reduced the yield of both extracellular and intracellular rinderpest virus in infected cells. Western blot analysis of both vLAV and vRBOK-infected cells revealed that the biosynthesis of two out of three major virus-induced polypeptides was affected by the presence of ammonium chloride. The estimated molecular weights of these two polypeptides strongly suggest them as H and F_1, which were reported to be glycosylated (Grubman *et al.*, 1988). On the other hand, another major immunological polypeptide, presumably the "nucleocapsid" protein with an estimated molecular weight 65 K, remained more or less unaffected by the presence of ammonium chloride. It has been reported for HSV-1 that while the glycolysation of several glycoproteins was affected in the presence of ammonium chloride, the viral nucleocapsid formation was not reduced at all (Kousoulas *et al.*, 1983; Koyama and Uchida, 1989). The results of our experiments on Western blot analysis of infected cells are also in agreement with those mentioned above. Weak bases elevate the intralysosomal pH resulting in the inhibition of uncoating processes of several RNA viruses (Marsh *et al.*, 1981). However, unaltered production of rinderpest nucleocapsid protein in the presence of ammonium chloride rules out the possibility of inhibition of uncoating process of rinderpest virus.

Amongst the possible mechanisms of action of ammonium chloride, the interference with the intracellular transport of the envelope glycoproteins through Golgi complex has been suggested (Matlin, 1986). Monensin, a carboxylic ionophor, was also shown to reduce both extracellular and intracellular yields of many enveloped viruses by affecting proton gradients in Golgi apparatus resulting in interference

with the trafficking of glycoproteins and egress of virions (Pressman, 1976; Kaarinainen *et al.*, 1980). Inhibition of HSV-1 replication by ammonium chloride has been indicated to occur by a mechanism similar to monensin (Koyama and Uchida, 1989).

Our experiments suggest that ammonium chloride inhibits its replication of rinderpest virus and affects biosynthesis of two putative viral glycoproteins possibly by interfering with the glycosylation process. Further experiments involving electron microscopy of infected cells and application of monoclonal antibodies should be undertaken not only to establish the mechanism of action of ammonium chloride more clearly but also to elucidate the phenomena of maturation and egress of rinderpest virus from infected cells.

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