

Coronavirus IBV-induced membrane fusion occurs at near-neutral pH

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Summary. The lysosomotropic agent NH_4Cl caused a reduction of 80–95% in the number of chick kidney (CK) cells and Vero cells infected by infectious bronchitis virus (IBV) strain Beaudette, as determined by immunofluorescence at the end of the first replication cycle. Inhibition only occurred when NH_4Cl was present during the first 2 h after infection. Syncytium formation was studied during replication of IBV-Beaudette in Vero cells. Some cell–cell fusion occurred at pH 7.0 and pH 6.5 but it was optimal at pH 6.7. IBV strain UK/123/82 did not replicate in Vero cells and was studied in CK cells in which it grew well but without forming syncytia. In contrast to IBV-Beaudette, NH_4Cl had virtually no effect on the replication of UK/123/82. The results show that the IBV spike glycoprotein induces membrane fusion at near neutral pH although some IBV strains may require a mildly acidic environment for the efficient uncoating of the virion RNA.

Introduction

Members of the monogeneric family *Coronaviridae* are enveloped, each species containing a large spike glycoprotein (S) which forms the bulbous surface projections, responsible for induction of membrane fusion [4, 20, 23], and a small membrane glycoprotein (M), most of which is not exposed at the outer virus surface. In IBV and some other coronaviruses the S protein is cleaved to form an amino-terminal S1 and a carboxy-terminal S2 subunit [1, 22]. A subset of the coronaviruses, which includes bovine coronavirus (BCV) and murine hepatitis virus (MHV) but not avian IBV, contains a third glycoprotein (HE) which has haemagglutinating and acetyl-esterase activities [22]. The ribonu-

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cleoprotein (RNP) comprises a single, positive-sense RNA molecule of some 30,000 nucleotides surrounded by a phosphorylated nucleocapsid protein (N) [22].

The study of membrane fusion induced by coronaviruses has been limited largely to MHV and BCV. Host cells infected with either virus fused to form syncytia when the culture medium was at pH 7.5 or greater but not at pH 7.0 or less [6, 8, 21]. These results indicated that the membrane fusing activity of the spike glycoprotein (S) did not require triggering in an acidic environment, in contrast to orthomyxoviruses, togaviruses and flaviviruses [8, 9, 10, 12]. Moreover, the results suggested that release of the RNP might occur following fusion of the virion envelope with the cell surface (plasmalemma) which can occur with paramyxoviruses [12, 16] and many mammalian retroviruses [14]. Indeed, fusion of BCV with the plasmalemma has been observed [19].

Fusion of coronaviruses with cell membranes has also been studied indirectly using lysosomotropic agents, for example NH_4Cl and chloroquine. These agents raise the pH of endosomes and lysosomes, in which viruses are located following endocytosis, and would be expected to inhibit the release of the RNP from an acid pH-requiring virus but not of a virus that was able to fuse at neutral pH. In agreement with the observations that BCV induced syncytia above pH 7.0 and fused with the plasmalemma, NH_4Cl had little effect on the replication of BCV [18, 19]. However, NH_4Cl and chloroquine did inhibit [11] or delay [15] the replication of MHV3 and MHV-A59, respectively.

To investigate further the conditions of coronavirus-induced membrane fusion we have examined the effect of acidic pH and NH_4Cl on the formation of syncytia and the early stages of infection, respectively, with IBV.

Materials and methods

Viruses and cells

IBV-Beaudette, adapted for growth in Vero cells, was as previously described [1]. Strain UK/123/82 had been passaged several times in chicken embryo tracheal organ cultures, and five times in embryos and primary chick kidney (CK) cells. Vero cells (ATCC No. CCL 81; Flow Laboratories, Irvine, Scotland) and CK cells were grown in M199 (Flow) and Eagle's minimal essential medium (MEM; Gibco, Paisley, Scotland) respectively, supplemented with 10% foetal calf serum, 0.22% sodium bicarbonate and penicillin and streptomycin, in 5% CO_2 . IBV-Beaudette was titrated by plaque assay in Vero or CK cells. Cells were washed with MEM buffered with 20 mM N-[hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) and 0.08% sodium bicarbonate. Infected cells were overlaid with MEM containing 10% tryptose phosphate broth (Difco) in place of serum, 1% agar (Difco) and sodium bicarbonate at 0.22% and 0.15% for CK and Vero cells respectively. After 3 days at 37°C a second overlay was added which contained 0.003% neutral red. Plaques were counted one day later. UK/123/82 did not form plaques and was titrated in tracheal organ cultures [3] and the titre expressed as \log_{10} median ciliostatic doses (CD)/ml.

Maintenance of cells at various pH

In order to maintain pH at desired values cells in closed flasks were used in an air incubator at 38°C. Maintenance medium comprised MEM, 0.2% bovine serum albumin (Boehringer-

Mannheim), 1.5 mg/ml tryptose phosphate (Difco) and was buffered by 20 mM N.N.bis[2-hydroxyethyl]-2 aminoethane sulfonic acid (BES; Sigma). Different amounts of a 1 M solution of sodium bicarbonate were added to give the desired pH, in the range 6.0–7.5, at 38 °C. The pH values in the flasks fluctuated within 0.1 pH unit during 48 h of incubation.

Virus replication in the presence of NH₄Cl

Experiments with NH₄Cl (FSA Laboratory Supplies, England) were performed in flasks or dishes. NH₄Cl in BES-buffered MEM, pH 7.0, was added to cells for 30 min prior to infection. Thereafter it was present as described in the Results section.

Immunofluorescence

Chicken anti-IBV strain M41 serum (20 ml) was absorbed three times using unfixed monolayers of Vero cells. The globulin fraction was obtained by two precipitations with 45% saturated ammonium sulphate and subsequently used at a concentration equivalent to a 1 : 2 dilution of the original serum. Cells on coverslips were fixed in ice-cold acetone for 10 min and then covered with antiserum for 1 h at room temperature. After washing this was replaced with rabbit anti-chicken IgG-fluorescein isothiocyanate (Bio-Yeda) for 1 h after which time the cells were permanently wetmounted for fluorescence microscopy [5].

Results

IBV-Beaudette-induced Vero cell syncytia developed optimally at pH 6.7

The Vero cell-adapted Beaudette strain of IBV produced large, distinct syncytia in Vero cells which were larger and remained on the substrate for much longer periods than did syncytia of CK cells. Consequently Vero cells in combination with IBV-Beaudette were chosen for the study of IBV-induced cell fusion.

In some pH experiments medium in the range pH 6.0 to 7.5 with 0.5 log₁₀ pH intervals was used. In one such experiment cells were infected at a multiplicity of infection (m.o.i.) of 0.0002 pfu/cell and incubated at 38 °C. Cell fusion was first observed at 24 h after infection but only at pH 6.5, there being 5 to 10 nuclei in the syncytia. 12 h later there was some fusion at pH 7.0 but it was less than at pH 6.5 (Fig. 1 A). In a similar experiment 0.25 log₁₀ pH intervals were used in the range pH 6.5 to 7.0 and at pH 7.5. After 36 h fusion was four-fold higher at pH 6.75 than at either pH 6.5 or 7.0 (Fig. 1 B).

To ascertain whether low pH had an irreversible, adverse affect on IBV infectivity, virus was diluted 100-fold with MEM at pH 5.0, 6.0, and 7.0 and held at 37 °C for 15 min. Titration showed that the titre of all three samples was within the range 5.3 to 5.5 log₁₀ pfu/ml.

Effect of NH₄Cl on the replication of IBV-Beaudette in Vero and CK cells

In order to determine if uncoating of the IBV genome required low pH in endosomes, the lysosomotropic agent NH₄Cl was used to raise the endosomal pH. To assess whether NH₄Cl would have any effect on the replication of IBV-Beaudette, Vero and CK cells were preincubated with 0, 5, or 10 mM NH₄Cl at 37 °C for 30 min. Both cell types were then inoculated with the same dilution

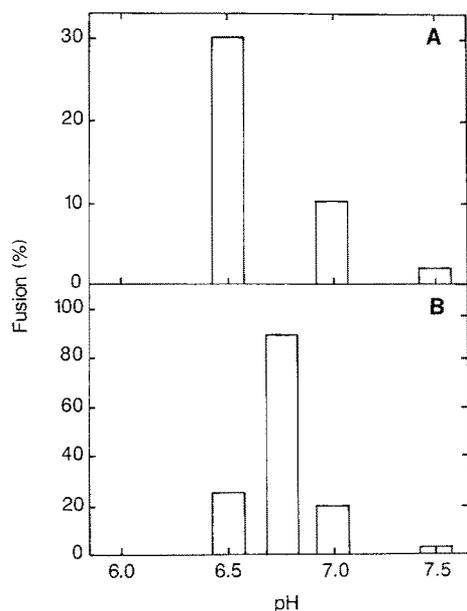


Fig. 1. Effect of pH on the induction by IBV-Beaudette of syncytium production in Vero cells. Fusion was observed at 36 h after infection at a moi of 0.0002 pfu/cell. **A** pH range was 6.0 to 7.5 in 0.5 log₁₀ steps; **B** range was 6.5 to 7.0 in 0.25 log₁₀ steps, plus pH 7.5

Table 1. Titre (log₁₀ pfu/ml^a) of IBV-Beaudette after incubation for 12 h in CK or Vero cells in the presence of NH₄Cl

Multiplicity of infection	Cell type	Concentration of NH ₄ Cl (mM)		
		0	5	10
0.3	CK	5.3	4.4	3.7
0.003	Vero	4.6	4.1	3.5

^a Titrated in Vero cells

of virus, with or without NH₄Cl, as appropriate. For CK cells this represented a m.o.i. of 0.3 pfu/cell (as titrated in CK cells) but only 0.003 pfu/cell for Vero cells (as titrated in Vero cells) as the titre of IBV-Beaudette was always about 100-fold higher when titrated in CK cells as compared with Vero cells. After 1.5 h the cells were washed and incubation continued at pH 7.0 with appropriate concentrations of NH₄Cl, samples being withdrawn at 12 h after infection, about the end of the first replication cycle, for assessment of the virus titre. Table 1 shows that in Vero cells 5 and 10 mM NH₄Cl reduced the virus titre by 32% and 92%, respectively, and in CK cells by 88% and 98%, respectively.

NH₄Cl affected an early stage in IBV replication

Further experiments were designed to identify whether NH₄Cl affected an early or late stage in infection. When IBV-Beaudette had been incubated, in the absence of cells, with 30 mM NH₄Cl at 37 °C for 1 h and subsequently titrated

in Vero cells, the titre (2.8×10^5 pfu/ml) was virtually the same as that of virus incubated without NH_4Cl (3.3×10^5 pfu/ml). Thus NH_4Cl had not had any direct virucidal effect on the virus.

Previous observations had shown that the IBV infection spread through Vero cell cultures largely by enlargement of the initial sites of infection, to form groups of infected cells, rather than by the establishing of new foci of infection. Viral proteins were detectable by fluorescence in the adjacent cells (second replication cycle) by 13 h after infection. Thus the number of groups of fluorescing cells essentially reflected the number of cells infected by the inoculum while the total number of fluorescing cells included those that had become infected by virus released after the first cycle of replication.

To determine if NH_4Cl would affect the attachment of virus to cells, Vero cells were held at 4°C for 30 min with virus in the presence of 30 mM NH_4Cl to permit attachment but not entry of the virus into the cells. The cells were then washed and incubation continued in the absence of NH_4Cl at 37°C for 13 h after which time the cells were fixed and examined using fluorescent antibody. The number of groups of infected cells (45) following attachment in the presence of NH_4Cl was virtually the same as without NH_4Cl (51); the total number of cells was 164 and 175, respectively. Thus NH_4Cl had not decreased the attachment of the virus to cells.

Further experiments were performed to determine whether NH_4Cl exerted its affect on IBV-Beaudette at an early, but post-attachment, or late stage in the infection cycle. In one experiment Vero cells were infected with IBV-Beaudette (m.o.i. 0.03) and incubated for a total of 14 h with NH_4Cl present or absent as shown in Table 2, experiment 1. The cells were then fixed and the infected cells detected using fluorescent antibody. Table 2 shows that the presence of NH_4Cl before infection and from 2 to 4 h after infection had no effect on the number of groups of cells which had produced virus proteins. In contrast, the presence of NH_4Cl during the first two hours of infection reduced the number of cells successfully infected by the inoculum by approximately 95%. The intensity of fluorescence of successfully infected cells was similar, irrespective of when NH_4Cl had been present. A second experiment (Table 2) confirmed the results of experiment 1, NH_4Cl having reduced the number of groups of infected cells by approximately 80% when present during the first 2 h of infection. In both experiments, for groups 1–4, the total number of infected cells was 3- to 6-fold greater than the number of cells which had been infected by the inoculum. This showed that following removal of NH_4Cl replication had proceeded normally. In the second experiment (Table 2) one group of cell cultures (group 5) had NH_4Cl present from 2 h until 14 h. In this case the number of cells productively infected by 14 h was virtually the same as the number of cells infected by the inoculum, i.e., the continued presence of the NH_4Cl had inhibited the second cycle of infection. The results showed that NH_4Cl had affected an early stage in the replication cycle.

Table 2. Effect of NH₄Cl (30 mM) on the infection of IBV-Beaudette in Vero cells, as assessed by immunofluorescence at 14 h after infection

Experiment no.	Group no.	NH ₄ Cl present (h after infection)				No. of groups of infected cells ^b	Total no. of infected cells ^b
		- 1/2-0 ^a	0-2	2-4	4-14		
1	1	+	+	-	-	2	11
	2	-	-	-	-	38	139
	3	+	-	+	-	31	180
	4	-	-	-	-	35	172
2	1	+	+	-	-	10	44
	2	-	-	-	-	58	365
	3	+	-	+	-	46	303
	4	-	-	-	-	52	358
	5	+	-	+	+	29	30
	6	-	-	-	-	32	289

^a NH₄Cl present 1/2 h before infection^b 50 fields examined

NH₄Cl did not affect replication of IBV strain UK/123/82

The replication of IBV UK/123/82 was studied in CK cells as this strain did not replicate in Vero cells. UK/123/83 did not induce syncytium formation. CK cells were pre-treated with NH₄Cl, at either 10 mM or 30 mM, for 30 min and then infected with either UK/123/82 or Beaudette at a moi of 0.1 CD₅₀/ml and 0.3 pfu/ml, respectively, in the presence or absence of the relevant concentration of drug. After 1 h the inoculum was replaced and the incubation continued with NH₄Cl present for the periods indicated in Table 3. At the end of the experiment the medium was harvested for virus titration. Table 3 shows that whereas 10 mM

Table 3. Effect of NH₄Cl on the replication of IBV strains UK/123/82 and Beaudette in CK cells, as assessed by virus titration

NH ₄ Cl present (mM)		Incubation time (h)	Infectivity (log ₁₀)	
10 ^a	30 ^b		UK/123/82 (CD ₅₀ /ml)	Beaudette (pfu/ml)
-		24	6.9	6.0
+		24	6.9	4.0
	-	10	5.7	3.1
	+	10	5.9	2.2

NH₄Cl present throughout^a or during only the first 2 h^b of infection

Table 4. Effect of NH₄Cl on the replication of IBV strains UK/123/82 and Beaudette in CK cells, as assessed by immunofluorescence at 10 h after infection

IBV strain	NH ₄ Cl (30 mM) – 1/2 to 2 h after infection	No. of infected cells in 30 fields
UK/123/82	+	301
UK/123/82	–	343
Beaudette	+	205
Beaudette	–	925

and 30 mM NH₄Cl resulted in a 100- and 10-fold decrease, respectively, in the titre of IBV-Beaudette, there was no effect on UK/123/82.

The effect of NH₄Cl on the first cycle of replication of UK/123/82 was further studied by immunofluorescence, the drug (30 mM) being present for 30 min before infection and for the first 2 h after infection. The CK cells had been grown on coverslips and were fixed at 10 h after infection. The results in Table 4 show that the drug decreased the number of cells productively infected with IBV-Beaudette by 78% whereas there was only a 13% decrease in the case of UK/123/82.

These results showed that NH₄Cl had had little or no effect on the release of the RNP of UK/123/82 and showed that the drug did not have any significant effect on any other stage of infection. This strengthened the view that the NH₄Cl had inhibited the infection of IBV-Beaudette in a specific manner, most probably during genome uncoating.

Discussion

Two membrane fusion events have been addressed. Firstly, the fusion of an infected cell expressing the S protein at its surface with neighbouring cells, to form syncytia. Secondly, the fusion of virion envelopes with cell membranes, as examined by raising the pH within endosomes with NH₄Cl. Although the pH conditions optimal for these two processes need not be the same, our observations on syncytium formation in Vero cells and the lack of an inhibitory effect of NH₄Cl on UK/123/82 replication in CK cells lead to the same conclusion, namely that the S protein of IBV is fusogenically active at around neutral pH. NH₄Cl can raise the pH of endosomes to almost pH 7.0 [24]. The finding that NH₄Cl only partially inhibited the replication of IBV is in keeping with our finding that Beaudette-induced syncytium formation did occur at pH 7.0 but was less extensive than at pH 6.7 (Fig. 1). The increased endosomal pH may have resulted in a reduction in the rate of membrane fusion such that some virions were transferred to and destroyed in lysosomes. The failure of NH₄Cl to inhibit replication of IBV UK/123/82 shows that fusion of this virus with cell membranes, leading to genome uncoating, occurred at about pH 7.0.

Thus a drop in pH, such as occurs within endosomes, was not required for membrane fusion with this virus, allowing the possibility that uncoating could occur while the virus was at the plasmalemma. Fusion of BCV virions (Mebus strain L9) with the plasmalemma, but not endosomal membranes, of HRT-18 cells has been observed by electron microscopy [19]. In addition NH_4Cl only reduced the output of BCV to a small degree (35%), leading to the conclusion that BCV genome uncoating did not require acid pH and that it occurred at the cell surface. Electron microscopic analysis of the entry of IBV-Beaudette into chicken chorioallantoic membrane cells and CK cells showed entry by phagocytosis and micropinocytosis [2, 17]. There was no evidence for fusion of IBV-Beaudette at the plasmalemma although fusion within vesicles was not observed either. Whether fusion occurs at the cell surface or within a vesicle, even with a virus for which an acidic environment is not required, will depend on the rate of fusion relative to the speed of endocytosis [13].

Further evidence supporting the view that the fusion activity of the S protein of some coronaviruses does not require low pH is provided by the observation that BCV-induced syncytium formation did not occur at pH 7.0 or below [18]. A pH of about 6.8 prevented syncytium induction, but not virus replication, by MHV-A59 whereas above pH 7.0 cell-cell fusion was extensive [21]. Vaccinia virus-expressed S protein of feline infectious peritonitis virus (FIPV) [23] and MHV-JHM [20] also caused cell-cell fusion in standard bicarbonate-buffered media. However, Vero cell syncytia induced by IBV-Beaudette clearly formed optimally at just under pH 7.0.

The location of the amino acid differences between the S proteins of IBV strains Beaudette and UK/123/82 which are responsible for the different pH requirements for membrane fusion activity might be investigated by producing various hybrid S genes and determining the pH requirement of the subsequently expressed proteins. Gallagher et al. [7] have very recently used this approach to study variants of MHV4 which, like the two IBV strains described herein, differed in their pH requirements for membrane fusion and susceptibility to NH_4Cl .

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