Mechanism of Uncoating of Influenza B Virus in MDCK Cells:
Action of Chloroquine

By MOTOHIRO SHIBATA,1 HIIZU AOKI, TATSUYA TSURUMI, YASUO SUGIURA,2 YUKIHIRO NISHIYAMA, SAKAE SUZUKI,1 AND KOICHIRO MAENO*

Department of Microbiology, Germ-free Life Research Institute, 1Department of Pediatrics, and 2Department of Anatomy, Nagoya University School of Medicine, Showa-ku, Nagoya, Aichi, Japan

(Accepted 8 December 1982)

SUMMARY
Exposure of influenza B virus-infected MDCK cells to chloroquine at the time of infection resulted in significant inhibition of infection. The appearance of input virus in the intracellular vesicles was not affected in the presence of the drug, but primary transcription of the virus genome did not occur. Chloroquine caused a rapid rise in the pH inside the lysosomes of MDCK cells, to 6.5 from the physiological pH 5.6. In contrast, exposure of infected cells incubated in acidic medium (pH 6.0) to chloroquine did not cause an increase in lysosomal pH and this low pH treatment during the chloroquine-sensitive phase was followed by virus production. Influenza B virus induced haemolysis of chick erythrocytes at low pH values (5.0 to 5.9) which was associated with cell–cell membrane fusion. It is likely that chloroquine prevents the uncoating of influenza B virus by increasing the lysosomal pH above the critical value required for inducing fusion between the virus envelope and the lysosomal membrane.

INTRODUCTION
Sendai virus exhibits haemolysis and fusion at neutral pH and can therefore transfer its genetic materials by envelope fusion with the target cell plasma membrane (Homm & Ohuchi, 1973; Scheid & Choppin, 1974, 1977), whereas other enveloped viruses generally enter the cells by endocytosis. Recently, Semliki Forest virus (SFV), vesicular stomatitis virus (VSV) and influenza A viruses have been found to cause haemolysis and fusion at low pH (Väänänen & Kääriäinen, 1979; Maeda & Ohnishi, 1980; White et al., 1980, 1981; Huang et al., 1981; Lenard & Miller, 1981; Matlin et al., 1981) and we have also observed similar activity of influenza B viruses at low pH values ranging from 5.0 to 5.9 (Shibata et al., 1982). These observations open up a new aspect of the mechanism of infection by these viruses.

Extensive studies with SFV have suggested that there is a fusion between the viral envelope and the membrane of the lysosomes into which the virus has been delivered, thereby releasing the nucleocapsid into the cytoplasm (Helenius et al., 1980). The fusion is thought probably to be triggered by low pH in the lysosomes, since lipophilic amines such as chloroquine that raise the pH within the lysosomes of macrophages inhibit the release of the virus genome into the cytoplasm. Similar evidence has been obtained recently with an influenza A virus (Matlin et al., 1981; Yoshimura et al., 1982).

In the present study, we examined the relationship between the effect of chloroquine on influenza B virus growth and the lysosomal pH of host cells. This paper presents evidence that influenza B virus may release its nucleocapsid into the cytoplasm by low pH-induced fusion between the virus envelope and the lysosomal membrane.

METHODS

Virus, virus assay and cell culture. Influenza B/Kanagawa/73 was used throughout the present study. Virus was grown in the allantoic cavity of 11-day-old chick embryos at 34 °C for 2 days. Haemagglutinin (HA) titration was
performed as described by Maeno & Kilbourne (1970). Madin and Darby canine kidney (MDCK) cells were grown in Eagle's minimum essential medium (MEM) containing 10% calf serum.

Infection and protein synthesis. MDCK cell monolayers (10⁶ cells/3 cm dish) were inoculated with influenza B virus at an input multiplicity of approximately 10 EID₅₀. After adsorption at 4 °C for 30 min, the cells were washed and incubated at 35 °C in serum-free MEM (pH 7:2).

At appropriate times after infection, the culture medium was removed and replaced with 15 μCi/ml [¹⁴C]methionine (New England Nuclear; 0-5 mCi/mmol) in MEM lacking unlabelled methionine. The radioactive polypeptides were examined by 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography (Laemmli, 1970; Maeno et al., 1979).

Fluorescence probe measurement of the intralysosomal pH. The pH inside the lysosomes of MDCK cells was determined essentially according to the methods of Ohkuma & Poole (1978). Briefly, fluorescein isothiocyanate–dextran (FD) was dissolved in 10 mm-phosphate or -acetate buffer containing 50 mM-NaCl at various pH values (4-0 to 7-5) to give 1 μg/ml, and the fluorescence at each pH was measured with a Hitachi fluorescence spectrophotometer 650-105 in the ratio mode. The excitation wavelength was 400 nm to 500 nm and emission was measured at 519 nm. The standard pH curve was prepared by estimating the ratio of fluorescence measured with excitation at 495 nm to that with excitation at 450 nm (FD 495/450 ratio).

Monolayers of 10⁶ MDCK cells in 50 cm² tissue culture flasks were incubated in MEM containing 1 mg/ml FD for 48 to 72 h after which FD was observed by fluorescence microscopy to be distributed in the cytoplasm in a pattern corresponding with that of the lysosomes. The cells were then rinsed with Hank's salt solution, scraped with a rubber policeman and suspended in phosphate-buffered saline (PBS), pH 7-2, at 36 °C. The excitation spectra of the cell suspensions were measured and the lysosomal pH value was estimated from the standard curve.

Extraction of virion RNA (vRNA) and preparation of ¹²⁵I-labelled vRNA. After clarification of the allantoic fluid, influenza B virus was pelleted at 20000 rev/min for 1 h and suspended in PBS. The virus suspension was layered on a 10 to 40% (w/v) linear sucrose density gradient in PBS and centrifuged in a SW27 rotor for 1 h at 20000 rev/min. The visible virus band was collected, resuspended in PBS and pelleted at 20000 rev/min for 1 h in a SW27 rotor.

Virion RNA was extracted three times with 1 vol. phenol–chloroform (1:1), precipitated from the final aqueous phase with 2 M-LiCl for 16 h at 4 °C and dissolved in distilled water. The amount of RNA was calculated assuming that one A₂₆₀ unit is 40 μg/ml (Seo et al., 1977).

¹²⁵I-labelled vRNA was prepared from influenza B virion by the thallium chloride procedure of Terebe & McCarthy (1973).

Extraction of cellular RNA. Mock-infected or influenza B virus-infected cells were harvested 3-5 h after infection. The extraction of cellular RNA was carried out according to the method of Seo et al. (1977).

RNA–RNA hybridization. The RNA preparations separately extracted from uninfected and infected cells were serially diluted in 30 μl distilled water, mixed with 20 μl 0-1 m-Tris–HCl pH 7-4, containing 0-75 m-NaCl, 5 mM-EDTA, 0-05% SDS, and ¹²⁵I-labelled vRNA and held at 68 °C for 160 h. Each mixture was then split into two parts, one being treated with S, nuclease at 40 °C and the other left at 40 °C. Acid-precipitable radioactivity was precipitated with 10% trichloroacetic acid (TCA) and assayed in an Aloka Auto Well Gamma System.

Electron microscopic examination. Cells were fixed with 1-5% paraformaldehyde and 1-5% glutaraldehyde in phosphate-buffered OsO₄. They were dehydrated in alcohol and embedded in Epon 812; ultrathin sections were stained with uranyl acetate and examined in a Hitachi 11-DS electron microscope.

Chemicals. Chloroquine (mol. wt. 515-9), S, nuclease, FD and actinomycin D were purchased from Sigma, and Na¹²⁵I (100 mCi/ml) from Amersham International.

RESULTS

Effect of chloroquine on influenza B virus growth in MDCK cells

Fig. 1 shows a dose–response curve of chloroquine on the yield of influenza B virus from MDCK cells. The virus was allowed to attach to the cells at 4 °C for 30 min. The cells were incubated in MEM (pH 7-2) containing the drug for 10 h at 35 °C. The drug completely inhibited the production of HA at a concentration of 100 μg/ml, but chloroquine (100 μg/ml) did not cause direct inactivation of the infectivity of influenza B virus (data not shown). Fig. 2 shows the time dependence of chloroquine inhibition. The addition of drug (100 μg/ml) before infection at 35 °C inhibited the production of HA, but this inhibitory effect was decreased when the drug was added later than 15 min after infection at 35 °C, indicating that chloroquine acts at a very early stage of virus infection. In the next experiment, the reversibility of chloroquine inhibition was investigated (Fig. 3). The medium was removed from chloroquine-treated cultures 1 h after infection at 35 °C and the cells were further incubated in normal medium up to 10 h after
Uncoating of influenza B virus

Fig. 1. Dose–response curve of chloroquine on influenza B virus yield in MDCK cells. The cells were pretreated with different concentrations of chloroquine for 1 h at 35 °C. Virus was inoculated without the drug at 4 °C for 30 min at an input m.o.i. of 10 EID_{50}. The cells were further incubated in 1 ml MEM with chloroquine for 10 h at 35 °C. Cells and culture fluids were frozen and thawed, and assayed for HA activity.

Fig. 2. Time dependence of chloroquine inhibition. Influenza B virus was adsorbed to MDCK cells at 4 °C for 30 min at an m.o.i. of 10 EID_{50}, after which cells were incubated in 1 ml MEM at 35 °C. Chloroquine (100 μg/ml) was added to the cultures at the indicated times before or after infection at 35 °C. Cells and culture fluids were harvested 10 h after infection, frozen and thawed, and assayed for HA activity. The broken line shows HA yield in untreated cells.

Fig. 3. Influenza B virus multiplication in chloroquine-treated, infected cells following removal of chloroquine. After adsorption at 4 °C for 30 min at an m.o.i. of 10 EID_{50}, infected MDCK cells were incubated at 35 °C in 1 ml MEM with (●, □) or without (○) 100 μg/ml chloroquine. At 1 h after infection at 35 °C, the medium was removed from chloroquine-treated cultures and the cells were further incubated in 1 ml MEM without chloroquine (●). At the indicated times after infection, cells and culture fluids were frozen and thawed and assayed for HA activity.

Fig. 4. Annealing of 125I-labelled vRNA of influenza B virus to RNAs from chloroquine-treated and untreated infected cells. After virus adsorption at 4 °C for 30 min at an m.o.i. of 10 EID_{50}, 10^6 infected MDCK cells were incubated in MEM with or without chloroquine (100 μg/ml) at 35 °C for 3.5 h. Increasing amounts of RNA from each of these cultures, or mock-infected cells, were mixed with 20 μl aliquots of 125I-labelled vRNA in a total vol. of 50 μl. A 20 μl sample of each mixture was annealed in duplicate for 160 h at 68 °C and each sample was further divided into two equal parts. One was treated with S1 nuclease, the other not, and RNase-resistant radioactivity was recorded as a proportion of the amount of RNA used. After hybridization of labelled vRNA with over 0.25 μg/ml of RNA from untreated, infected cells, the RNase resistance was 71%. ○, Chloroquine-treated, infected cells; ●, untreated, infected cells; ■, mock-infected cells.
Fig. 5. Effect of chloroquine on the translation of primary transcripts of influenza B virus. After adsorption at 4 °C for 30 min at an m.o.i. of 10 EID₅₀, infected cells were incubated in MEM containing cycloheximide (100 μg/ml) at 35 °C for 4 h. The medium was then removed and the cells were labelled for 15 min with 15 μCi/ml [³⁵S]methionine in MEM lacking unlabelled methionine (lane 1). Chloroquine (100 μg/ml) was added to some of these cultures at the time of infection (lane 2) or 30 min after infection (lane 3), and the cells were pulse-labelled with [³⁵S]methionine 4 h after infection. Cycloheximide-treated, infected cells were also labelled for 15 min after removal of cycloheximide at 30 min after infection (lane 4). Whole cells were subjected to electrophoresis on a 10% polyacrylamide slab gel, followed by autoradiography. Unlabelled virion polypeptides were also run in parallel as markers. Migration is from top to bottom. Mock-infected cells are in lane 5.

Fig. 6. Chick erythrocyte-chick erythrocyte fusion mediated by influenza B virus at low pH. Four ml of allantoic fluid (256 HA units per 0.25 ml) from influenza B virus-infected chick embryos was mixed with 1/10 vol. of a 5% chick erythrocyte suspension and allowed to stand at 4 °C for 30 min. Erythrocytes were pelleted by centrifugation at 1000 rev/min for 5 min, washed, and suspended in 0.1 M-PBS pH 7.2 (a) or 5.9 (b). The suspension was kept in a 36 °C water bath for 30 min and centrifuged at 1000 rev/min for 5 min; the erythrocytes were processed for electron microscopic examination. Bar markers represent 1 μm.

Infection at 35 °C. In untreated cells, HA activity was detected at 4 h after infection and reached a maximum at 8 h, but none was synthesized in the presence of drug. However, removal of the drug from the cultures readily restored the production of HA, with a 2 h delay (Fig. 3).
Uncoating of influenza B virus

The effect of chloroquine (100 μg/ml) on virus entry into MDCK cells was examined by electron microscopy. After virus adsorption at 4°C for 30 min, infected cells were incubated at 35°C in MEM with or without chloroquine. Before warming the cultures, all virus particles remained attached to the cell surface. After warming up the untreated cultures to 35°C, a large number of the virus particles appeared in intracellular vesicles within 5 min (data not shown). These events were unaffected by chloroquine, indicating that chloroquine does not alter the normal route of virus entry.

Effect of chloroquine on primary transcription of the virus genome

Influenza B virus-infected cells were incubated in MEM with or without chloroquine (100 μg/ml) for 3-5 h after infection at 35°C and RNA was extracted from each of these cultures.

Increasing amounts of the RNA were hybridized with 20 μl aliquots of 125-I-labelled vRNA from influenza B virions and the RNase-resistant radioactivity was measured. As shown in Fig. 4, labelled vRNA was protected from RNase by hybridization with increasing amounts of RNA from untreated, infected cells but not with the same amount of RNA from treated, infected cells, indicating that the synthesis of influenza B virus complementary RNA (cRNA) does not occur in the presence of chloroquine.

We examined whether chloroquine inhibited transcription of the infecting virus genome. Influenza B virus-infected cells were incubated at 35°C in MEM containing cycloheximide. At 4 h after infection, the medium was removed and the cells were pulse-labelled for 15 min with [35S]methionine, followed by SDS-PAGE and autoradiography. Significant amounts of the viral polypeptides HA, nucleoprotein (NP), membrane protein (M) and non-structural protein (NS) were detected (Fig. 5, lane 1). However, when chloroquine was added at the time of infection at 35°C, no detectable amounts of these viral polypeptides were synthesized (Fig. 5, lane 2). When chloroquine was added 30 min after infection, the viral polypeptides were detected in significant but reduced amounts (Fig. 5, lane 3). When cycloheximide-treated infected cells were washed at 30 min post-infection and pulse-labelled for 15 min, they did not synthesize detectable amounts of viral polypeptides, except for NP in a reduced amount (Fig. 5, lane 4). These results were not changed by the addition of actinomycin D (1 μg/ml) 30 min before pulse-labelling (data not shown), suggesting that the viral polypeptides synthesized under the above conditions were the translation product of the primary transcripts. Actinomycin D completely inhibited the growth of influenza B virus in MDCK cells when added within 2 h after infection, as observed with influenza A virus (Rott et al., 1965). The above results indicate that chloroquine does not inhibit the activity of virion-associated transcriptase of infecting virus, suggesting that chloroquine inhibits infection by acting at a step preceding the primary transcription of the influenza B virus genome.

Interaction between chick erythrocyte and influenza B virus at low pH

Influenza B virus induces extensive haemolysis at low pH values, from 5·0 to 5·9 (Shibata et al., 1982). Chick erythrocytes agglutinated by the virus were incubated in saline buffered to pH 7·2 or 5·9 at 36°C for 30 min and examined for virus-cell membrane interaction by electron microscopy. At pH 7·2 a number of virus particles were seen outside or on the surface of erythrocytes, all of which appeared morphologically normal and did not fuse with each other (Fig. 6a). In contrast, at pH 5·9 all erythrocytes tested appeared to liberate haemoglobin and exhibited membrane fusion between two cells (Fig. 6b). Neither virus-like particles nor fusion between viral envelope and cell membrane could be observed. When these erythrocytes were pelleted, the supernatant exhibited no HA activity, in contrast to control supernatant in which 100% of original HA activity was recovered (data not shown). These results suggest fusion of the virus envelope with the cell membrane. This haemolysis reaction at pH 5·9 was unaffected by the addition of chloroquine (data not shown). The agglutinated erythrocytes were incubated in pH 5·9 saline at 36°C for 1 to 5 min but no fusion between virus envelope and cell membrane could be observed by electron microscopy.
Fig. 7. Effect of chloroquine on the pH inside the lysosomes of MDCK cells. The cells containing FD in their lysosomes were washed with Hanks' balanced salt solution and suspended in PBS pH 7.2 in the fluorescence cell. (a) Chloroquine (100 µg/ml) was added to the suspension. Ten min later, the drug was removed from the cell suspension and the cells were suspended in PBS pH 7.2 (W). After an additional 10 min incubation, chloroquine was again added to the cell suspension (C). (b) The cells containing FD in their lysosomes were suspended in PBS pH 7.2 in the fluorescence cell. Chloroquine was added to the cell suspension. Ten min later, the cells were washed and resuspended in PBS pH 6.0 containing 100 µg/ml chloroquine (arrow). The pH was calculated from the FD 495/450 ratio.

*Effect of chloroquine on the pH in the lysosomes of MDCK cells*

A pH-sensitive fluorescent probe was employed to measure the lysosomal pH of a suspension of living MDCK cells held in PBS (pH 7.2), and the kinetics of the pH change was quantitatively determined by fluorescence spectroscopy (see Methods). Fig. 7(a) shows the effect of chloroquine on the pH in the MDCK cell lysosomes. The normal pH inside the lysosomes was around 5.6. This value was within the pH range optimal for the haemolysis reaction by influenza B virus (Shibata et al., 1982). When chloroquine was added to the culture, the lysosomal pH rapidly increased to 6.5, which is above the critical value required for haemolysis by influenza B virus. This lysosomal pH dropped back to a value close to its initial value immediately after the removal of the drug from the medium. The same result was also observed when MDCK cells were tested 1 h after infection (data not shown). These findings indicate that extracellular chloroquine is essential for the maintenance of elevated pH inside the lysosomes.

Further experiments were carried out to confirm that elevation of the lysosomal pH by chloroquine is responsible for its inhibition of infection. After virus adsorption at 4 °C for 30 min, infected cells were incubated at 35 °C in chloroquine-containing MEM buffered to pH 6.0 with 1 mM-sodium phosphate for 15 min and the medium was then replaced with normal medium containing the drug. The cultures were assayed for HA after 10 h. Incubation of infected cells in normal medium with the drug resulted in no production of HA, but a brief exposure to chloroquine in pH 6.0 medium permitted the production of HA to a level comparable to that in cells incubated in drug-free medium. Addition of chloroquine to MDCK cells cultured at pH 6.0 did not cause an increase of lysosomal pH (Fig. 7b). It is unlikely that fusion between virus envelope and the plasma membrane initiated infection, since the virus could not induce haemolysis at pH 6.0.

**DISCUSSION**

Influenza B virus penetrated the cells by endocytosis and rapidly appeared in the intracellular vesicles. Virus entry was unaffected by the addition of chloroquine at or before the time of infection, but infection was inhibited. However, the drug had no effect on virus growth when added 15 min after infection.
Uncoating of influenza B virus

Chloroquine had no ability to inhibit the activity of the virion-associated transcriptase of infecting virus, but the transcription of the virus genome was inhibited by the addition of the drug at the time of infection. These results suggest that chloroquine inhibits infection by acting at a replicative step preceding primary transcription of the virus genome. Similar conclusions have also been reported for the effect of another lipophilic amine, amantadine, on influenza A viral replication (Kato & Eggers, 1969; Skehel et al., 1977; Koff & Knight, 1979).

The pH in the lysosomes is acidic, and lipophilic amines accumulate in the lysosomes of macrophages and elevate the lysosomal pH (Ohkuma & Poole, 1978). Chloroquine also had a similar effect on the pH in the lysosomes of MDCK cells which was raised to 6.5 from the physiological pH of 5.6. This elevation of the lysosomal pH by the drug seemed to be responsible for the inhibition of the infection. The increase in pH we observed was higher than that reported for MDCK cells by Yoshimura et al. (1982), possibly because these authors used monolayers rather than cells in suspension. Exposure to chloroquine of cells incubated in acidic medium (pH 6.0) did not cause an increase in the lysosomal pH, and such treatment at a chloroquine-sensitive step permitted virus multiplication. Poole et al. (1979) indicated that under acidic conditions lipophilic amines cannot pass through the cell membrane.

Influenza B virus induced fusion between the cell membranes of erythrocytes at pH values ranging from 5.0 to 5.9. A possible explanation for the inhibitory effect of chloroquine is that influenza B virus releases its ribonucleoprotein into the cytoplasm by low pH-induced fusion between the virus envelope and the lysosomal membrane and that this fusion reaction is inhibited in the presence of chloroquine, due to the high pH environment inside the lysosomes. This notion is supported by evidence that influenza B virus with uncleaved HA has neither low pH-induced haemolytic activity nor infectivity (Shibata et al., 1982). Recent studies with SFV, VSV, and influenza A (fowl plague) virus have suggested that low pH-dependent fusion plays an important role in the infective process (White et al., 1980, 1981; Matlin et al., 1981; Yoshimura et al., 1982). It is interesting to note that low pH-induced fusion may occur between virus and endosome membrane as well as, or more efficiently than, with the lysosomal membrane. Recent work has demonstrated that endosomes differ from classical secondary lysosomes by the absence of acid hydrolases. Nevertheless, they have relatively acidic contents (Geisow et al., 1981; Geisow, 1982). If so, virion uncoating can take place without the degradation of virus particles by the lysosomal hydrolases.

Tryptic cleavage of HA of influenza virus into the HA1 and HA2 polypeptides results in activation of low pH-dependent fusion and haemolytic activities (Huang et al., 1981; Maeda & Ohnishi, 1980; Shibata et al., 1982). The HA2 N-terminal segment containing 10 or more sequential hydrophobic amino acid residues is responsible for these activities (Richardson et al., 1980; Garten et al., 1981) and is located near the base of the HA spike (Wilson et al., 1981). Recently it has been suggested that a conformational change induced in the HA molecule by low pH treatment may contribute to the interaction of the hydrophobic segment of HA, with the cell membrane (Skehel et al., 1982). Alternatively, HA aggregation by HA-HA interaction at low pH may occur in the virus membrane, with subsequent modification of the membrane structure leading to the exposure of membrane lipid.

We thank E. Iwata and T. Tsuruguchi for their excellent technical assistance. This work was supported by research grants from the Ministry of Education, Science and Culture of Japan.

REFERENCES


(Received 11 August 1982)