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Short communication

Effect of bafilomycin A1 on the growth of Japanese encephalitis virus in Vero cells

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We studied the effect of bafilomycin A1 (Baf-A1), a novel and highly specific inhibitor for vacuolar-type proton (V-H$^+$) pump, on the growth of Japanese Encephalitis virus (JEV) in Vero cells. Viral fluorescence microscopic study showed that Baf-A1 induced the complete disappearance of acidified compartments such as endosomes and lysosomes in Vero cells by the treatment with 0.1 μM Baf-A1 for 1 h at 37°C. In proportion to the disappearance of acidified compartments, virus growth was inhibited when Baf-A1 was present from 1 h before infection to the end of incubation in a dose-dependent manner, or added within as early as 5 min after infection. Conversely, the virus growth was recovered in correlation with the reappearance of acidified compartments after removal of Baf-A1. These results suggest that a low pH condition, which is regulated by Baf-A1-sensitive V-H$^+$ pumps, is essential for the early stage of JEV growth.

Keywords: flavivirus; bafilomycin A1; antiviral activity; vacuolar-type proton pump; acid catalyzed fusion reaction

Japanese encephalitis virus (JEV) is one of the important etiological agents for central nervous diseases and/or encephalitis (Monath and Heinz, 1996). JEV, a member of the family Flavivirdae, has a positive-sense and single-stranded RNA genome of about 11 kilobases in length (Hashimoto *et al*, 1988; Sumiyoshi *et al*, 1987). Recently, it has been shown that envelope glycoprotein spikes of flavivirus exhibits acid-catalyzed fusion reaction like influenza virus (Heinz *et al*, 1994; Helenius, 1995; Rey *et al*, 1995). However, the role of this phenomenon on the JEV growth process has not yet been completely understood. In the case of influenza virus, the acidic condition in endosomes and lysosomes (referred to as ELS in this study) is essential for the uncoating process by triggering the viral envelope fusion activity (for an in-depth review, see White *et al*, 1983). Indeed, chloroquine, an endosomotropic weak base, inhibits the growth of influenza A and B viruses by raising the pH within ELS (Shibata *et al*, 1983; Ochiai *et al*, 1995). On the other hand, several studies indicate that the ELS membranes contain vacuolar-type proton (V-H$^+$) pumps, which are, at least in part, responsible for the acidification of ELS (Ohkuma *et al*, 1982; Forgac *et al*, 1992). Ochiai *et al* (1995) have shown that influenza virus growth is inhibited in proportion to the disappearance of acidified ELS induced by treatment with bafilomycin A1 (Baf-A1), a novel and highly specific inhibitor for vacuolar-type proton (V-H$^+$) pump (Bowman *et al*, 1988; Hanada *et al*, 1990; Werner *et al*, 1984). In addition to study the role of acidified ELS on the virus growth process, the use of Baf-A1 provides an opportunity to study whether acidification of ELS is regulated by the contribution of additional proton transfer systems induced in response to virus infection by overcoming the disadvantage of chloroquine (Ochiai *et al*, 1995). In light of these findings, we have adopted the use of Baf-A1 to clarify the role of acidic condition in ELS on the JEV growth.

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JEV, JaGAr-01 strain was used throughout the study. Confluent monolayer of Vero cells, a monkey kidney cell line, in a 24-well plate was infected with JEV at a multiplicity of infection (MOI) of five plaque forming units (PFU)/cell and cultured for 30 h at 37°C. Thereafter, the cultures were processed three times of freezing and thawing and centrifuged at 1000 r.p.m. for 10 min to assay virus yields in the supernatants by the plaque method on BHK-21 cells. The culture condition of the cells, preparation of virus solution and plaque assay method were described previously (Takegami *et al*, 1990).

Initially, the effect of Baf-A1 on the acidified ELS in Vero cells were examined by vital fluorescence staining (Yoshimori *et al*, 1991) with acridine orange, an acidotropic weak base, which is taken up by living cells and changes its color from green at low concentrations to orange at the accumulated site such as acidic ELS (Allison and Young, 1969; Holtzman, 1989). After treatment with various doses of Baf-A1 for 1 h at 37°C (short-term treatment), the staining cells were observed with the aid of a fluorescence light microscopy (Figure 1). Numerous acidified compartments with a dotted distribution could be observed in the drug-untreated cells. Their number was decreased in the drug-treated cells in a dose-dependent manner. At 0.1 and 0.08 μM, Baf-A1 inhibited completely and partially the acidification of ELS, respectively, whereas the effect of 0.04 μM Baf-A1 was negligible. In the cells treated with 0.1 μM Baf-A1, only diffused green fluorescence was observed clearly in the nuclei and faintly in cytoplasm by the staining of nucleic acids with the dye.

As shown in Figure 2, the growth of JEV was inhibited in the successive presence of drug from 1 h before infection to 30 h post infection (p.i.) in a dose-dependent manner. In the control culture supernatants, virus titers were detected at a level of more than 10^6PFU/ml, whereas these values decreased proportionally in the drug-treated cells at doses of 0.05 to 0.2 μM Baf-A1. Growth inhibitory effect of Baf-A1 was negligible at a dose of less than 0.04 μM. These results show that the doses of Baf-A1 on the inhibition of virus growth coincides with those on the inhibition of ELS acidification.

To obtain a better insight into the drug effect, reversibility and time-related drug effect were

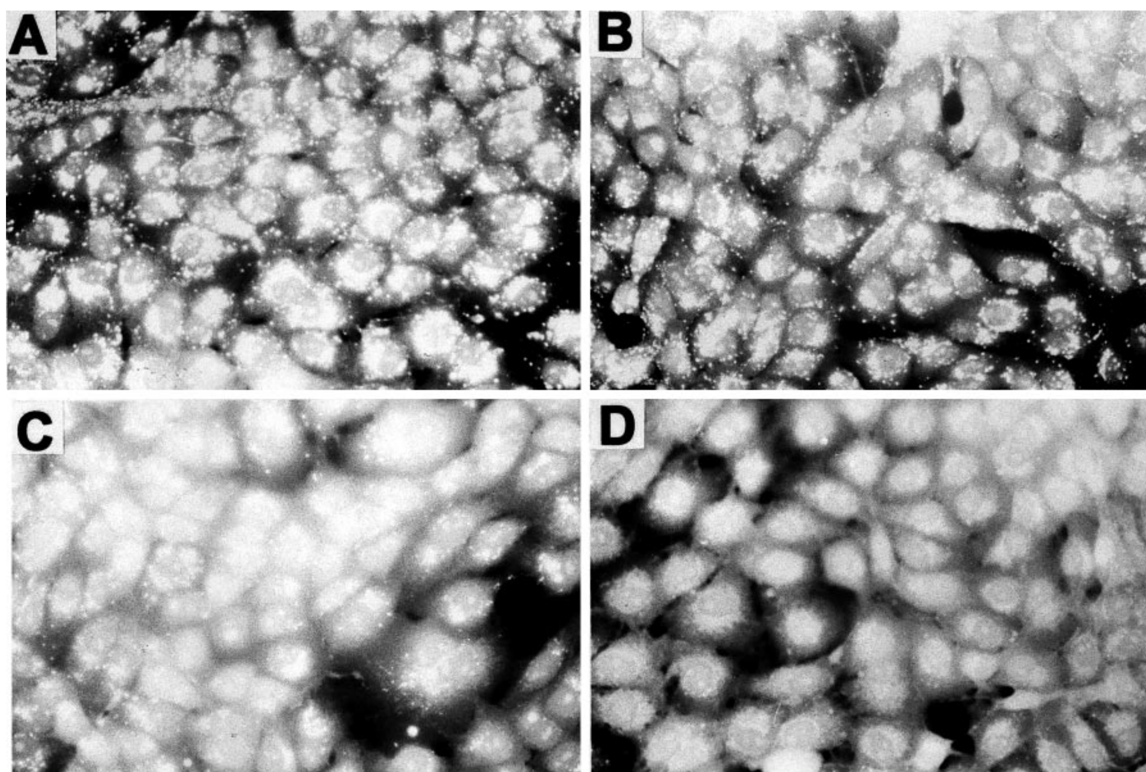

Figure 1 Effect of Baf-A1 on the acidification of ELS in Vero cells. Confluent monolayer of the cells on the cover slips was washed once with phosphate-buffered saline (PBS) and then treated with 0.04 μM (B), 0.08 μM (C) or 0.1 μM (D) Baf-A1, or not treated (A) in serum-free media for 1 h at 37°C. Thereafter, the cells were washed three times with serum-free media followed by vital fluorescence staining for 10 min at 37°C with acridine orange. After washing as above, the cells were observed with the aid of fluorescence microscopy (Olympus BX50). The representative figures of 3 experiments are presented. Bar represents 20 μm.

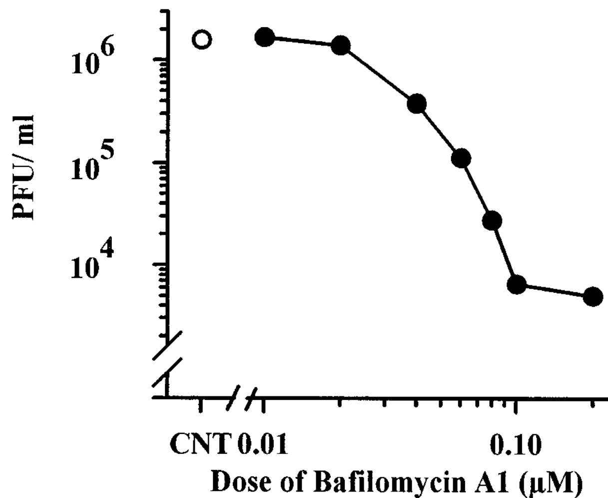

Figure 2 Effect of Baf-A1 on the growth of JEV in Vero cells. Confluent monolayers of the cells was washed once with PBS and then treated with various doses of Baf-A1 (0.01 to 0.2 μM expressed by a log scale in the abscissa) for 1 h at 37°C. The pretreated cells were infected with JEV for 1 h at room temperature followed by washing three times as above and then cultured for 30 h at 37°C in the presence of the same drug doses as for the previous treatment. Thereafter, the cells were processed three cycles of freezing and thawing to examine virus yields by plaque assay. As a control (CNT in the abscissa), the cells were processed throughout in the drug-free condition. Open and closed circles indicate the virus yield in the control and drug-containing cultures, respectively. The representative data of three experiments are presented.

studied. To examine reversibility of drug effect (Figure 3), growth curve was compared among various conditions for drug treatment such as only pre-treatment with drug (0.1 μM) for 1 h before infection followed by culture in a drug-free condition, or both pre- and post-treatment in the same manner as above. In sharp contrast to drug-untreated culture (control) in which virus titers reached to a considerably high level (more than 10^5PFU/ml) at 12 h p.i., the virus growth was apparently inhibited in both pre- and post-treated culture in which virus titer was retarded to a maximum 10^3PFU/ml at as late as 30 h p.i. Compared with the growth curve of control, virus growth was delayed with a time-lag of 12 h in the culture receiving only pre-treatment, resulting in an intermediate growth curve between the former two conditions. These findings show that the drug effect is reversible by culture in drug-free condition, and internalized virus particles under drug treatment might be protected from hydrolytic degradation in lysosomes containing acidophilic enzymes as reported by Yoshimori *et al* (1991). Indeed, acidification of ELS in Vero cells recovered with a time-lag of 2 h after removal of drug (0.1 μM) (Figure 4). Furthermore, it was investigated whether drug (0.1 μM) affects the early phase of JEV infection. Figure 5 shows that the virus growth was inhibited only by the

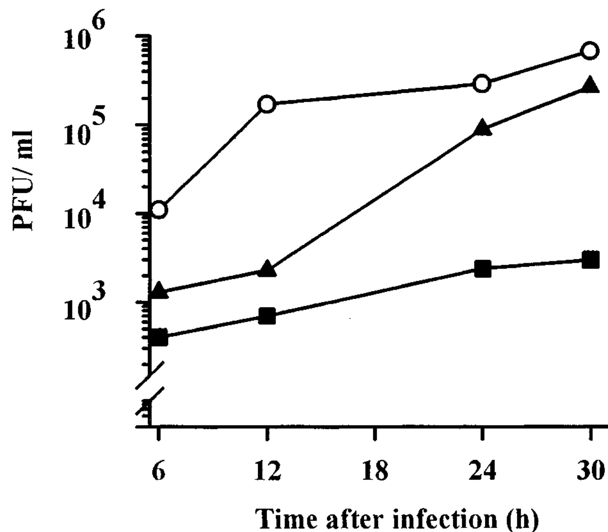

Figure 3 Reversible effect of Baf-A1 on the growth of JEV in Vero cells. The cells were previously treated with Baf-A1 (0.1 μM) and then infected with JEV. After adsorption, the pretreated cells were further cultured in the presence (closed square) or absence (closed triangle) of drug. At 30 h p.i., virus yields in the culture supernatants were determined. As a control, the cells were processed throughout in the drug-free condition (open circle). The methods for cell preparation, virus infection and the assay on the virus yields are described in the legend in Figure 2. The representative data of three experiments are presented.

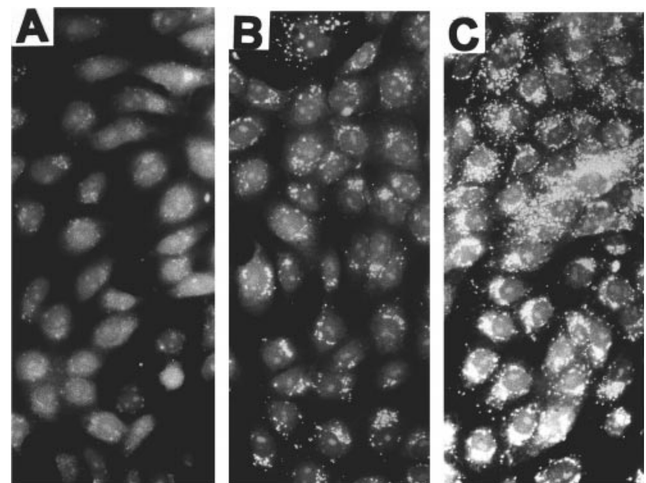

Figure 4 The recovery of acidification of ELS in Vero cells. A confluent monolayer of the cells on the cover slips was washed once with PBS and then treated with 0.1 μM Baf-A1 in serum-free media for 1 h at 37°C. After removing the drug-containing media, the cells were washed once with serum-free media and then cultured in a drug-free condition. The acidification of ELS was observed at 0 h (A), 1 h (B) and 2 h (C) after replacement to drug-free condition as described in the Figure 1. The representative data of three experiments are presented. Bar represents 20 μm.

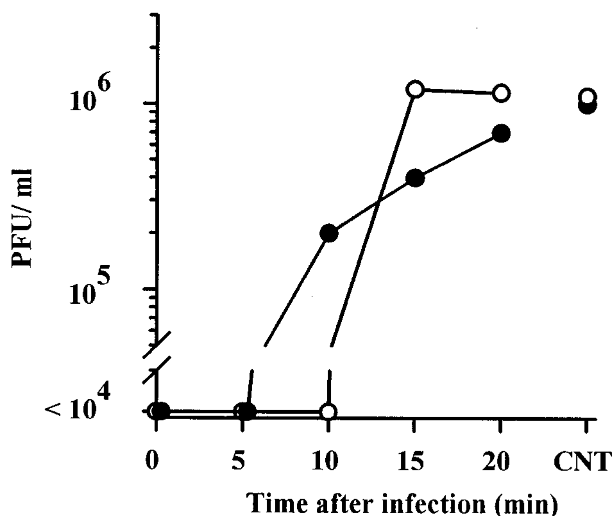

Figure 5 Time-related drug effect on the growth of JEV in Vero cells. Confluent monolayers of the cells were infected in the drug-free condition. After adsorption, Baf-A1 (0.1 μM; closed circle) or chloroquine (100 μg/ml; open circle) was added to the culture media at 0, 5, 10, 15, and 20 min p.i.. At 30 h p.i., virus yields in the culture supernatants were determined. The control cells were processed throughout in the drug-free condition (CNT in the abscissa). The methods for cell preparation, virus infection and the assay on the virus yields are described in Figure 2. The representative data of three experiments are presented.

initiation of drug treatment within as early as 5 min p.i. but not by the subsequent onset. The pattern of time-related drug effect was almost similar to that of chloroquine (100 μg/ml), confirming that both drugs affect the early phase of JEV infection.

As to cytotoxic activity of Baf-A1, morphological changes (cell atrophy and nuclear picnosis) and detachment of Vero cells were actually induced in a short-term treatment with 1 μM Baf-A1. However, in a long-term treatment (30 h) with 0.1 μM drug, cytotoxic activity could be ruled out by Trypan blue exclusion test and regrowth assay in which the growth of drug-treated cells were monitored after replacement to drug-free media (data not shown).

In summary, this study demonstrated that Baf-A1 inhibits the growth of JEV showing a strict relation with the acidification of ELS, indicating that the acidified compartments are essential for the early phase of JEV infection. Our results also suggest that Baf-A1-sensitive V-H^+ pumps are solely responsible for the acidification of ELS, eliminating the possibility of the induction of Baf-A1-insensitive proton transfer systems in JEV-infected cells. Baf-A1 might be a useful tool to study the uncoating process via acid-catalyzed fusion reaction in JEV infection.

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