The requirement of environmental acidification for Ibaraki virus infection to host cells

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ABSTRACT. The effect of environmental acidification on Ibaraki virus (IBAV) infection was tested using endosomal inhibitory chemicals and low pH treatment. Treatment of target cells with endosomal inhibitors significantly decreased the progeny virus production. IBAV outer capsid proteins, VP5 and VP2, were removed from virion when purified IBAV was exposed to low pH environment. Further experiment showed that the exposure to low pH buffer facilitated IBAV infection when the cellular endosomal pathway was impaired by bafilomycin A1. Results obtained in this study suggest that acidic environment is essential to initiate IBAV infection.
KEY WORDS: acidic environment, endocytosis, IBAV infection

Ibaraki virus (IBAV) is one of the strains of epizootic hemorrhagic disease virus (EHDV) serotype 2, a member of genus *Orbivirus* in *Reoviridae* family [11, 19]. It is known as a causative agent of Ibaraki disease characterized by the paralysis of swallowing muscles. Although the effective vaccine is widely used, the recent reemergence of Ibaraki disease in combination with the co-circulation with other EHDV raised the alert level for the epidemic of Ibaraki disease [13]. In addition, orbivirus family contains many viruses causing severe loss in the livestock industry. Therefore, it is important to understand the detailed molecular mechanism of their infection.

Virus infection is initiated by the release of viral genome to the intracellular compartment of target cells. Several mechanisms are known for the genome release to cytoplasm, and one of the most well known mechanisms is the direct fusion mechanism utilized by several enveloped viruses, such as human immunodeficiency virus-1, mouse hepatitis virus and Paramyxoviruses [5, 6]. Enveloped viruses are also known to use host endosomal pathway to facilitate their infection [12].

Reovirus family does not have envelope, and its infection to host cells is believed to occur via host endosomal pathway [2, 12]. Once attached to the cell surface, the host endosomal pathway incorporates particles. Along with the endosome maturation, the dissociation of outermost capsid protein and proteolytic cleavage on viral protein will occur [3, 4]. Especially, the proteolytic cleavage by endosomal proteases, cathepsin B and L, is an essential process for infection [1]. In other words, the expression level of those proteases affects the susceptibility of the host cells to virus infection. In addition to endosomal proteases, extracellular proteases also play an important role to promote certain reovirus infection *in vivo*. It was shown that tissue specific proteases contribute for the definition of target organ of rotavirus infection [10].

On the other hand, the critical condition to activate orbivirus infection remains unidentified. Although it has been suggested that orbivirus also utilizes host endosomal pathway, no involvement of protease was reported [8]. From studies with enveloped viruses, it was shown that viruses utilize endosomal pathway in two different ways. Some viruses, such as influenza virus, use low pH environment itself, and other viruses, such as severe acute respiratory syndrome virus, use endosomal proteases rather than low pH environment itself [12, 18]. To clarify which mechanism was used by orbivirus, we tested the effect of environmental pH for orbivirus infectivity using IBAV.

The utilization of endosomal pathway by IBAV for infection was confirmed using endosome inhibitors. HmLu-1 (hamster lung) cells were infected with IBAV in the presence of three different endosome inhibitors, bafilomycin A1 (Baf A1, Sigma, St. Louis, MO, U.S.A.), chlorpromazine (CPZ, Abcam, Cambridge, U.K.) and dynasore (Wako, Osaka, Japan). Those drugs inhibit clathrin dependent endocytosis in different manners. Baf A1 is an antibiotic derived from *Streptomyces griseus* and specifically inhibits vacuolar type H+ ATPase [23]. CPZ dislocates clathrin, and its adaptor protein from plasma membrane to cytosol [22] and dynasore inhibits GTPase activity of dynamin specifically [14]. All inhibitors work reversibly, and therefore, endosome pathway can restart once the drug was removed from the culture. HmLu-1 cells prepared in 6 well plate were treated with Dulbecco's modified eagle's medium (DMEM, Wako) containing various concentrations of inhibitors for 30 min at 37°C. After cells were chilled on ice for 5 min, the media

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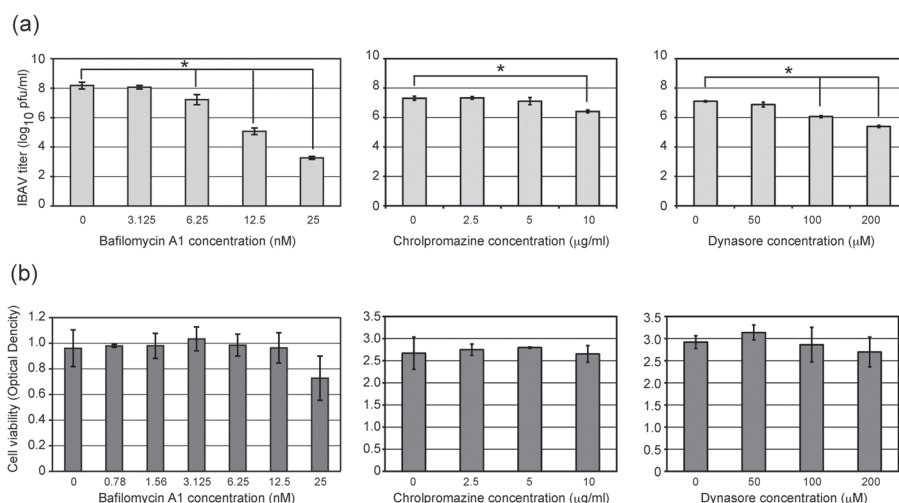 e-mail: r.nabewo@gmail.com

Fig. 1. Effect of endosome inhibitors on IBAV infectivity. (a) The amount of infectious IBAV in the supernatant from HmLu-1 infected with IBAV under the various concentrations of endosome inhibitors. Asterisks indicate groups with significant difference from control (no inhibitor) group. (b) Cell viability of HmLu-1 after being treated with various amounts of inhibitors. The result from Baf A1 treated cells is the representative of three independent experiments.

were removed, and cells were infected with IBAV at MOI=3 for 1 hr at 4°C. After a wash with PBS (−), cells were further incubated with DMEM plus inhibitors for 30 min at 37°C, and the media were replaced with DMEM containing 10% fetal bovine serum (10FDMEM). Cells were incubated for further 24 hr, and culture supernatant was collected. Collected supernatant was subjected to plaque assay, and after staining with crystal violet solution (0.1% crystal violet in 10% buffered formalin and 20% methanol), the number of plaque was counted. To analyze the statistical significance of each inhibitor concentration group against mock treated group, statistical software R [16] was used to run Dunnet's test [7]. Figure 1a shows the number of infectious IBAV in the supernatant of the cells treated with Baf A1, CPZ or dynasore. All inhibitors were shown to decrease virus titer when their concentration was elevated. The most significant decrease was observed when Baf A1 concentration was higher than 6.25 nM (P=0.0013 for 6.25 nM against 0 nM). At the same time, the effect of those inhibitors to HmLu-1 viability was tested. HmLu-1 cells in 96 well multiwell plate were treated with media containing various concentrations of inhibitors for 1 hr without IBAV infection. After 24 hr incubation, the number of viable cells was quantified using CellTiter® Aqueous One Solution Reagent (Promega, Madison, WI, U.S.A.). As shown in Fig. 1b, no inhibitors showed significant effect on viable cell numbers. These results indicated that IBAV utilizes clathrin-dependent endosomal pathway for infection and coincided with the previous research on bluetongue virus entry [8].

To confirm the effect of low pH on virus infectivity, purified IBAV was incubated in PBS (−) with several pH (pH=4, 7 or 9) for five min and infected to HmLu-1 at MOI=0.01. We employed lower MOI to protect cells from severe CPE at early time point and achieve multi cycle infection. DMEM
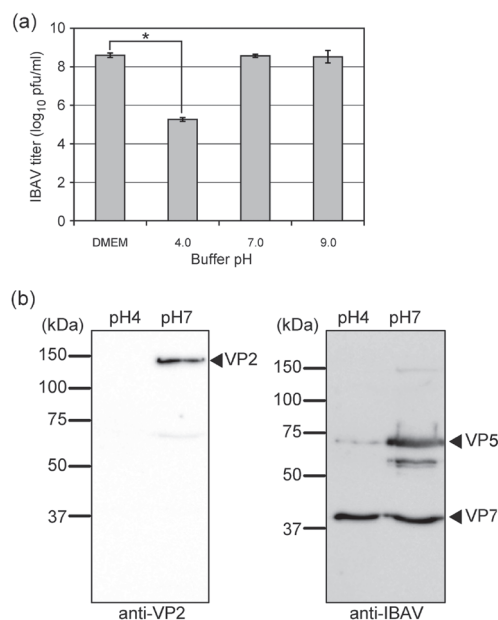

Fig. 2. Inactivation of IBAV by acidic condition. (a) The amount of IBAV in the supernatant from HmLu-1 infected with IBAV treated with PBS (−) with various pH indicated. The result is the representative of three independent experiments. Asterisk indicates significant difference from control (DMEM) group. (b) Western blot analysis of purified IBAV particle after pH4 or pH7 treatment. Mouse antisera raised against VP2 (anti-VP2) or purified IBAV particle (anti-IBAV) were used as first antibodies.

without FBS was used for control incubation, and the significance in the difference between control and each test group was analyzed by Dunnet's test. As shown in Fig. 2a, the pro-
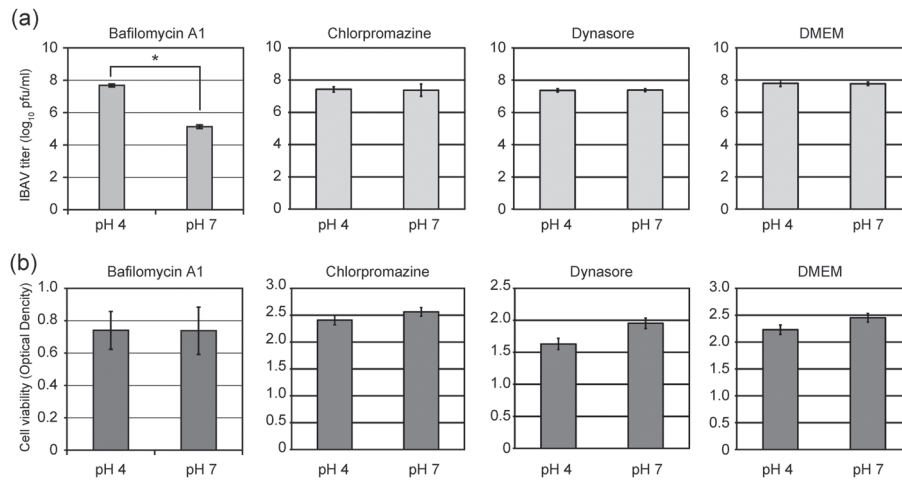
Fig. 3. Requirement for the exposure to the low pH environment for IBAV infection. (a) The amount of infectious IBAV in the supernatant from HmLu-1 attached with IBAV and treated with PBS (−) with indicated pH. 12.5 nM Baf A1, 10 $\mu g/ml$ CPZ and 200 μM Dynasore were used. Asterisk indicates the existence of the statistically significant difference between two treatment groups. (b) Cell viability of HmLu-1 after being treated with inhibitors and PBS (−) with indicated pH. The result from Baf A1 treated cells is the representative of three independent experiments.

duction of progeny virus was significantly suppressed when IBAV was treated with PBS (pH4) compared to control treatment ($P<0.001$). Other conditions did not give any impact on IBAV infectivity (pH7 and pH9, P value was 0.993 and 0.906, respectively). The pH of PBS-IBAV mix solution was confirmed with pH test paper after 5 min incubation (data not shown). Many enveloped viruses lose its infectivity by low pH treatment, if they utilize endosomal acidification to trigger the activation of their protein that is responsible for infection [15, 17, 18]. The result obtained in this study was consistent with these observations suggesting that low pH treatment caused irreversible change on IBAV particle that leads to the loss of infectivity.

To understand the mechanism of this loss of infectivity, virus particle was collected by ultracentrifugation after low pH treatment. Western blot analysis using anti-VP2 antisera and anti-IBAV antisera was performed, and VP2, VP5 and VP7 were visualized (Fig. 2b). The approximate molecular weights of VP2, VP5 and VP7 are 115, 60 and 38 kDa, respectively. In the case of BTV, it was shown that VP2 and VP5 form outer capsid layer and play important roles for the initial step of infection [24]. VP2 is a protein reported as a sialic acid binding protein and implicated to play a role in the attachment to target cells [24]. VP5 is believed to work as a protein which disturbs the lipid bilayer of target cells [9]. As shown in the left panel of Fig. 2b, VP2 was detected in the virus treated with PBS (pH7), whereas no VP2 was detected in the virus treated with PBS (pH4). Similarly, less VP5 was detected in the virus treated with PBS (pH4) compared to the virus treated with PBS (pH7). The amount of VP7 seemed to be the same between PBS (pH4) and PBS (pH7), and hence, it was suggested that there are the same amounts of viral core in each sample.

The result obtained above implicated that low pH treat-

ment removes IBAV outer capsid proteins from the particle and initiates its infection. To test if low pH treatment can by-pass the inhibitory effect of endosome inhibitors, we treated IBAV attached to cell surface with PBS (pH4) or PBS (pH7) under the presence of endosome inhibitors. Briefly, HmLu-1 was pretreated with inhibitors for 30 min. Cells were chilled on ice for 5 min and infected with IBAV at MOI=3 for 1 hr at 4°C. At this step, virus cannot get internalized by endocytosis, because of the low temperature. Attached viruses were treated with PBS (pH4) or PBS (pH7) for 5 min at 37°C, and cells were further treated with 10FDMEM plus inhibitor for 30 min at 37°C. After the 24 hr incubation with 10FDMEM, virus titer in the supernatant was determined. As shown in Fig. 3a, the significant difference was observed in the virus titer between PBS (PH4) and PBS (pH7) only when Baf A1 was used ($P=0.0036$, Welch's t-test). When cells were treated with other inhibitors, different pH of 2 buffers made no difference in viral titer. Figure 3b shows the viability of HmLu-1 after a series of treatment without virus infection, and no significant difference was observed ($P=0.983$, Welch's t-test).

Results obtained in this approach confirmed the usage of clathrin-dependent endocytosis pathway by orbivirus and support the previous report [8]. In addition, it was proved that the exposure to low pH environment is the essential condition to initiate IBAV infection. In the presence of Baf A1, the acidification of late endosome, as well as its fusion to lysosome is impaired [20, 21]. Although the cell surface viruses could be internalized during the post-treatment, they cannot be exposed to low pH environment in the presence of Baf A1. However, if viruses were exposed to low pH at the cell surface before internalization, they acquired the infectivity and could start infection under the effect of Baf A1. PBS (pH4) failed to enhance IBAV infection under the

influence of other two inhibitors. Since those two inhibitors interrupt endosome pathway at the very beginning, no IBAV could be incorporated during post-treatment. This might make both IBAV groups to start infection at the same time and resulted in the same viral titer. Since we could not deny an involvement of any proteases, additional research will be required to reveal the entire molecular mechanism of orbivirus infection. The importance of low pH environment for IBAV activation that we proved in this study will help to design experimental approach for further studies.

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