Short Communication

Plaque Formation by Japanese Encephalitis Virus Bound to Mosquito C6/36 Cells after Low pH Exposure on the Cell Surface

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SUMMARY: Japanese encephalitis virus (JEV) formed plaques in mosquito C6/36 cell layers after adsorption on the cell surface and exposure to pH values lower than 6.2. The number of plaques decreased within pH ranges from 7.4 to 6.4, but increased within pH ranges from 6.2 to 5.8. Plaque formation was prevented by treatment of the virus with a JEV-neutralizing monoclonal antibody, 503, after virus adsorption. Plaque formation was not affected by pretreatment with a specific V-ATPase inhibitor, bafilomycin A1. The results indicate that JEV successfully fused with the C6/36 cell membrane under acidic conditions below pH 6.2, which in turn led to plaque formation in C6/36 cell layers. These results suggest that productive JEV infection occurs at the C6/36 cell surface via the fusion between JEV and the cell membrane.

A number of studies provided evidence that flaviviruses infect target cells by endocytosis and low pH-induced fusion with endosomal membranes (1-5). There is also a process by which enveloped viruses gain early entry into their host cells by fusion between the viral membranes with cellular membranes (6). The pH-dependent fusion activity of flaviviruses has been demonstrated using model membranes to study both fusion from without and fusion from within; however, there is as of yet no evidence that fusion on the plasma membrane induces productive viral infection (7-9). The present study was conducted to investigate whether or not the fusion between JEV and the C6/36 cell membrane induces productive infection.

An Aedes albopictus mosquito cell clone, C6/36, was cultured in Dulbecco-modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS). Japanese encephalitis virus (JEV), a JaGAr01 strain, was propagated in C6/36 cells as previously reported (10). Virions were purified from culture supernatant fluids from infected cells, according to a previously described method (10). The infectivity titer of the virus preparation was 8.8 × 10⁶ plaque-forming units (pfu) per mg of protein in C6/36 cell cultures. The JEV envelope (E) protein-specific monoclonal antibody (MAb) 301 and MAb 503 were kindly provided by Dr K. Yasui, Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan (11,12). MABs were purified from mouse ascitic fluids using Protein G column chromatography (PIERCE, Rockford, Ill., USA). Mouse IgG (NM IgG) was used as the control. Unless otherwise stated, the data represent the mean of three to five independent assays and the standard error.

Fusion between JEV and the cell membrane was assessed by plaque formation after adsorption of the virus to the cells and exposure to an acidic pH on the cell surface. Cell-adsorbed virions were exposed to the buffer adjusted to pH ranges from 7.4 to 5.8 according to the hemagglutination (HA) titration method described by Clarke and Casals (13). In brief, 100 to 150 pfu/0.2 ml of the virus suspensions were prepared in DMEM supplemented with 2% FCS (pH 7.0 to 7.2), and these samples were inoculated to C6/36 cell layers on ice, which were then kept on ice for 1 h. Unabsorbed virus was washed three times using ice-cold Hank’s balanced salt solution (HBSS, pH 7.0 to 7.2). Virus-adsorbed cells were kept in a mixture of 0.5 ml of 0.05 M borate-saline (BS9, pH 9.0) and an equal volume of the acidic diluent (VAD) to yield pH ranges from 7.4 to 5.8. The JEV-inoculated cell layers were exposed to pH values ranging from 7.4 to 5.8 for 5 min at 31°C. In the preliminary experiments, the kinetics of JEV infection of C6/36 cells was examined at 31°C. Sixty-five to 80% of the plaques had formed within the first 10 min of incubation; therefore, 5 min was adopted as the incubation-time assay condition for this study (data not shown). The reaction was blocked by the addition of 150 µg of MAb 503 IgG (15 µg/0.1 ml of HBSS), and the samples were left to stand on ice for 30 min. After removal of the buffer, the cell layers were covered with 2.0 ml of agar overlay medium supplemented with excess amounts of MAb 503 IgG (150 µg), and the samples were cultured at 28°C in an atmosphere of 5% CO₂ for 5 to 6 days.

From 100 to 134 plaques were counted in C6/36 cell layers at pH ranges from 7.4 to 7.0, and the plaque numbers decreased in a linear manner at pH ranges from 6.8 to 6.4. The plaque number increased at pH ranges from 6.2 to 5.8 to pH levels from 7.4 to 7.0 (Fig. 1A, open column). Pretreatment of the virus with MAb 503 IgG inhibited plaque formation across all assay conditions (Fig. 1A, solid column). These results suggest that the uncoating of JEV occurred at the C6/36 cell surface due to the fusion between the viral and cellular membranes under acidic conditions, i.e., below pH 6.2. It was reported that (i) fusion reaction by cell-bound JEV was observed in C6/36 cells, and (ii) JEV fusion activity became apparent at pH values below 6.2 (14,15). The present results are consistent with those of previous studies of JEV fusion.

To confirm that the plaque formation by JEV was due to membrane fusion at the cell surface under the present experimental conditions, C6/36 cell layers were pretreated with a specific V-ATPase inhibitor, bafilomycin A1, in order to inhibit cellular endocytosis. C6/36 cell layers were treated...
with bafilomycin A1 at a concentration of 1.0 to 1.2 μM in maintenance medium (2% FCS-DMEM) for 1 h at 28°C (Fig. 1B), according to the method described by Mizutani et al. (4). Control cells were treated with dimethyl sulfoxide at final concentrations of 1.0 to 1.2% (v/v). The patterns of plaque formation are similar between bafilomycin A1-treated (open column) and mock-treated (screened column) cell layers; plaque numbers decreased linearly at pH levels ranging from 7.4 to 6.2, and increased again at levels below pH 6.0. Plaque density in the treated cells ranged from 63 to 69% of that in untreated cells across all assay conditions. These observations suggest that viral infection occurs through a process of membrane fusion on the cell surface via an endocytic pathway.

The effects of MAb 503 on the low pH-induced conformational changes of the E protein were examined (Fig. 2). JEV antigen-coated ELISA plate wells (1.0 μg of protein per well) were reacted with normal mouse IgG (solid diamonds) or MAB 503 at a concentration of 5.0 μg (open circles) and 2.5 μg (open squares) IgG per well. After incubation at 4°C for 10 min, and then the complexes were re-equilibrated with phosphate-buffered saline, pH 7.2. Changes in the E protein were assessed by adding the appropriate dilution of enzyme-conjugated MAb 301 IgG. A pH-dependent change in recognition by MAb 301 was influenced by pre-treatment of the E protein with MAB 503. Similarly, it was previously reported that Mab 301 and MAB 503 did not compete with each other in an ELISA-based competitive binding assay (12). The present results suggest that MAB 503 inhibited the conformation of the E protein at low pH levels, as detected by MAb 301.

The precise mechanism of acidic pH-triggered structural change in the JEV E protein has not been defined. However, it is known that the exposure of JEV to an acidic pH environment induces conformational changes in the E protein, leading to the fusion between the viral envelope and the cellular membrane. C6/36 cell-bound JEV led to productive infection after exposure to environments with pH levels below 6.2, thus suggesting the fusion between the virus and the cell membrane as a mechanism of infection. In a series of experiments conducted to examine the effects of pH treatment on the free-virus particle, virus titration was carried out in C6/36 and Vero cell layers. After exposure of the samples to an environment kept at pH 6.0 for 5 min, titers decreased by 10^{2.86} and 10^{5.57} pfu/ml in C6/36 and Vero cells, respectively (data not shown). This finding suggests that JEV primarily entered these cells via an endocytic pathway, whereas JEV can also enter mosquito C6/36 cells at the cell surface via membrane fusion. A fusion assay (i.e., fusion from without) was performed using cultured mosquito cells. However, it should be noted that the current methods of investigation are limited; virus-adsorbed cells were incubated at temperatures higher than 35°C, and then the samples were fixed and stained with appropriate stains (8,15). Further investigations are still needed to better define this mechanism; however, the results of the present study indicate that fusion between JEV and the C6/36 cell membrane leads to productive infection. The results also suggest that the binding of MAB 503 to the epitope interferes with the low pH-dependent conformational changes.
in the E protein that are essential for the entry of JEV into target cells, and subsequently to the low pH-induced fusion between JEV and the C6/36 cell membrane. In a related study, Morita et al. (16) previously mapped the epitope recognized by MAb 503 by using a long PCR-based region-specific random mutation technique. They showed that amino acid substitutions at positions 52, 126, 136, and 275 at the junction between domains I and II of the E protein eliminated the reactivity of JEV to MAb 503 (17). Accordingly, the most probable mechanism of virus neutralization by MAb 503 is the conformational changes induced during the fusion of the E protein with the target cell membrane.

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REFERENCES


