

Short Communication

Interference in Japanese encephalitis virus infection of Vero cells by a cationic amphiphilic drug, chlorpromazine

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Entry of Japanese encephalitis virus (JEV) into cells was analysed by using the vertebrate cell line Vero. Vero cells were treated with chlorpromazine, nystatin or cytochalasin D, which inhibit clathrin- and caveola-dependent endocytosis, and macropinocytosis of the cells, respectively. Productive JEV infection was inhibited by pretreatment with chlorpromazine; the number of JEV antigen-positive cells was less than one-fifth of that in untreated cultures, but was not significantly decreased by pretreatment with nystatin or cytochalasin. Viral antigens were detected in the membrane fractions, but not in the endosome fractions from chlorpromazine-treated JEV-inoculated cells. When the cells were treated with chlorpromazine, clathrin heavy chain antigen and JEV antigen were not detected in cytoplasm by indirect immunofluorescence staining. These results indicate that JEV is taken up by cells through the clathrin-dependent endocytic pathway, and this process leads to infection.

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Japanese encephalitis virus (JEV) is a member of the family *Flaviviridae*. Virus infection requires entry of the virus into the host cell. The viral genome can enter the cytoplasm by penetrating the plasma membrane at the cell surface, or at the endosome after endocytosis. The process of the early phase of flavivirus infection is not clearly understood; however, there are reports that flaviviruses enter the cell by receptor-mediated endocytosis (see review of Heinz & Allison, 2001). Clathrin-dependent and caveola-dependent pathways were both reported to be the virus entry mechanism (see review of Siczekarski & Whittaker, 2002). Endocytic activity of cells can be analysed by using pharmacological agents. Wang *et al.* (1993) demonstrated that chlorpromazine induced the assembly of clathrin lattices on endosomal membranes and at the same time prevented coated pit assembly on the cell surface of human fibroblasts. Nystatin is a sterol-binding agent and removes membrane cholesterol, which is required to maintain caveolae in the plasma membrane (Anderson, 1993; Lisanti *et al.*, 1993; Rothberg *et al.*, 1990). Cytochalasin D was used to inhibit macropinocytosis of the cell (Maniak, 2001). In the present study, we analysed the early phase of JEV infection in a vertebrate cell line, Vero. We attempt to address the question of whether JEV enters cells through the clathrin-dependent endocytic pathway. This study provides an insight into the interaction between JEV and vertebrate cells.

Vero cells were cultured in Dulbecco's modified Eagle's

medium (DMEM) supplemented with 10% foetal calf serum (FCS). JEV, JaGAR-01 strain, was propagated in *Aedes albopictus* cell clone C6/36, and purified from culture supernatants of infected cells according to the method described previously (Nawa, 1996). The titre of JEV was 3.4×10^{10} p.f.u. ml⁻¹. Entry of JEV into Vero cells in the infection process was analysed by detecting viral antigens in the cell. Vero cells growing on Lab-Tek II chamber slides (2.1×10^5 cells per well) were treated with chlorpromazine ($10 \mu\text{g ml}^{-1}$), nystatin ($10 \mu\text{g ml}^{-1}$) and cytochalasin D ($5 \mu\text{g ml}^{-1}$) for 1 h at 37 °C. Cells were then inoculated with JEV (6.7×10^7 p.f.u.) in the presence of the drug, and incubated at 37 °C for 4 h. After the first 4 h of the incubation period, cells were washed with Hanks' balanced salt solution (HBSS), pH 7.4, and incubated for a further 3 days in 10% FCS/DMEM in the presence of JEV-neutralizing, envelope (E) glycoprotein-specific mouse mAb 503 (Kimura-Kuroda & Yasui, 1986; 200 $\mu\text{g IgG}$ per well, 144 ND₅₀) or normal mouse IgG (200 $\mu\text{g IgG}$ per well). We selected mAb 503 because the JEV-neutralizing activity of mAb 503 is greater than the other JEV E protein-specific mAbs and the polyclonal antibody (Butrapet *et al.*, 1998). On day 4, cells were washed with PBS and then fixed with 4% paraformaldehyde in PBS. Virions and non-structural viral antigens present in the cells were detected by flavivirus cross-reactive human IgG purified from dengue patients' sera (Hu-IgG), and then with FITC-conjugated F(ab')₂ fragment of goat anti-human immunoglobulins (Zymed).

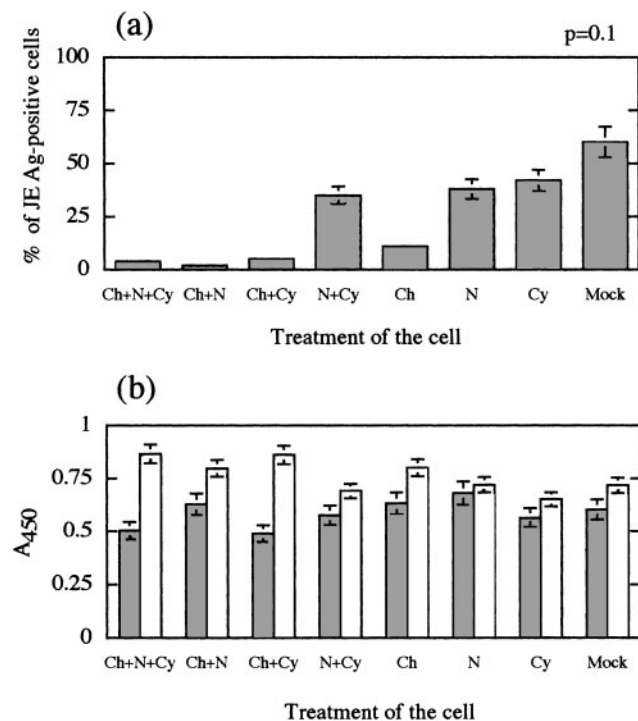


Fig. 1. Effects of drug treatment on productive infection with JEV. Vero cells were treated with chlorpromazine (Ch), nystatin (N) or cytochalasin D (Cy), according to the method described in the text. Images from the experiments were recorded for quantitative analysis on Fuji Superia 400 colour print film. For each experimental point, four fields or more were randomly chosen, and photographed with filter sets for FITC using a Nikon Fluophoto microscope and a 20 \times objective lens. (a) 100 cells were scored in each picture and the percentages of JEV antigen-positive cells were calculated. (b) Cell viability was measured by using the XTT assay kit according to the manufacturer's manual. Grey-filled columns and white columns indicate cell viability (measured as A_{450}) on the 2nd and 4th day of incubation, respectively. Data are means of two separate assays, and bars indicate standard error in each assay.

The percentage of JEV antigen-positive cells was 60% in the control cell culture, whereas in the cultures treated with chlorpromazine, nystatin and cytochalasin D it was 11, 35 and 42%, respectively (Fig. 1a). The percentages of viral antigen-positive cells were lower (2 to 5%) in the cultures treated with mixtures of chlorpromazine and the other two drugs. In the cell cultures treated with a mixture of nystatin and cytochalasin D, 35% of the cells were JEV antigen-positive.

The viability of the cells was examined after treatment with the drugs, using the commercially available cell proliferation kit II (XTT) (Roche) (Fig. 1b). Vero cell layers in a culture plate (96-well) were incubated for 4 days, and examined on days 2 and 4. Treatment of the cells with chlorpromazine, nystatin or cytochalasin D did not decrease the viability of the cells (Fig. 1b). Productive JEV infection was affected

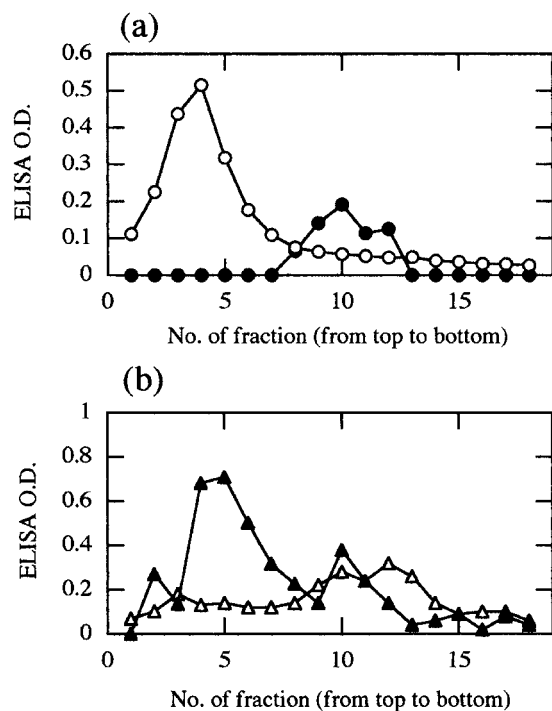


Fig. 2. Subcellular distribution of JEV in drug-treated Vero cells. The endosome (○) and the membrane (●) fractions are indicated in (a), and virion antigen in the control (▲) and the drug-treated (△) cells are indicated in (b). After centrifugation, the gradients (2.0 ml per tube) were fractionated to 18 tubes (0.1 ml per tube) from top to bottom. Each fraction was mixed with an equal volume (0.1 ml) of 0.2% Triton X-100/PBS, and then divided into two parts. One aliquot (100 μ l) was used to measure the protein contents by BCA protein assay reagent (Perbio Science), and the other aliquot was used to detect the antigen by ELISA. Data indicate ELISA O.D. (measured as the A_{492} per mg of protein) obtained with each fraction.

differently by chlorpromazine, nystatin and cytochalasin D without any difference in viability of the cell; the effect of chlorpromazine was greater than that of the other two drugs. Therefore, the inhibitory effect of chlorpromazine is not due to cell toxicity.

Endocytic activity was analysed in Vero cells by examining the uptake of JEV with human transferrin as a reference. Human transferrin is known to be taken up by the cells through clathrin-dependent endocytosis, but it does not enter lysosomes (van Renswoude *et al.*, 1982). Subcellular distribution of transferrin (Fig. 2a) and JEV (Fig. 2b) was analysed by Percoll density gradient centrifugation, according to the method described previously (Nawa, 1997). The FluoReporter cell-surface biotinylation kit (Molecular Probes) was employed to label cell-surface proteins, and used to identify the membrane fraction in the post-nuclear cell homogenate (Fig. 2a).

Vero cells growing in plastic culture dishes (60 mm in

diameter) were treated with chlorpromazine. JEV at a multiplicity of 1000 p.f.u. per cell or horseradish peroxidase (HRP)-conjugated human transferrin (10 μ g per dish; Rockland Inc.) and biotin-XX sulfosuccinimidyl ester (1 μ g per dish) was added to the wells, and the plates were kept on ice for 1 h. The cells were washed thoroughly

with ice-cold HBSS, and incubated for 15 min at 37 °C to allow the ligands to be taken up by the cells. Homogenization and subcellular fractionation were carried out, according to the method described previously (Nawa, 1997). Ligands in the fractions from the Percoll density gradient were detected by an antigen-capture ELISA. It was

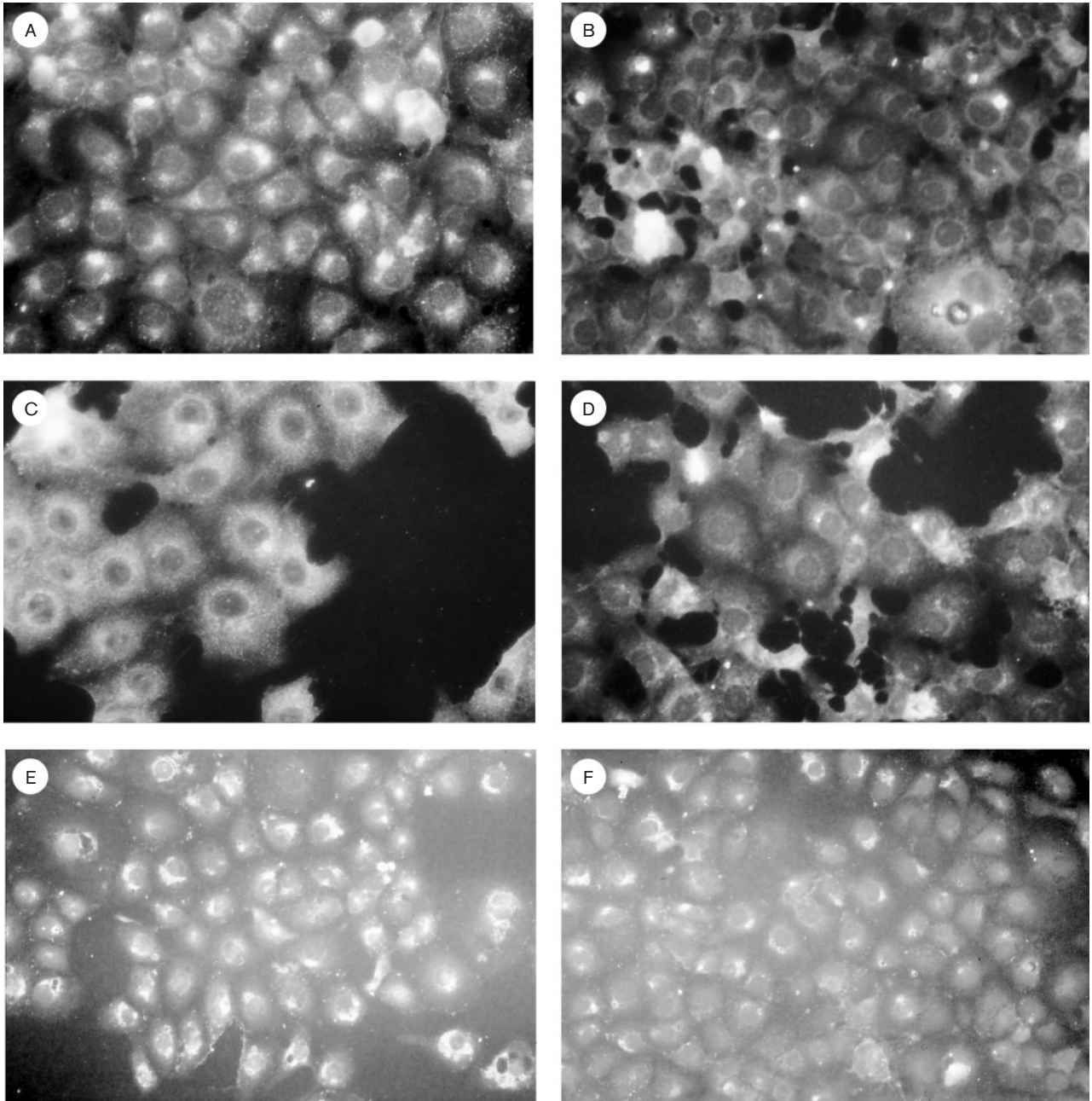


Fig. 3. Effects of chlorpromazine on uptake of JEV and intracellular distribution of clathrin in the cells. Vero cells were incubated in the absence (a, c and e) and presence (b, d and f) of 10 μ g chlorpromazine ml^{-1} at 37 °C for 1 h. The cells were then inoculated with JEV (a and b) at 37 °C for 30 min. Virus-inoculated (a and b) and mock-inoculated (c and d) cells were fixed with ethanol and reacted with the anti-flavivirus monoclonal antibody D1-4G2 IgG (a and b) and anti-clathrin heavy chain IgG (c and d). (e) and (f) show viral antigen production in the control (e) and chlorpromazine-treated (f) cells on day 4 after infection, according to the method described in Fig. 1(a).

previously reported that JEV was taken up into the acidic compartment of the cells during the entry process (Nawa, 1997), and antigenic reactivity of epitopes on the E protein was affected at the pH ranges corresponding to the acidic compartments of the cells (Nawa, 1996). Virions were captured on the solid phase with Hu-IgG ($20 \mu\text{g ml}^{-1}$). The HRP-conjugated human transferrin and biotinylated membrane proteins were captured on the solid phase of 96-well ELISA plates, which were coated with a diluted (1:500) anti-human transferrin goat serum (EY Lab. Inc., USA), and $10 \mu\text{g streptavidin ml}^{-1}$ (Vector Labs), respectively. Captured antigens were detected as follows: (i) HRP-transferrin was detected by adding the enzyme substrate solution (0.5 mg ml^{-1} *o*-phenylenediamine and 0.1% H_2O_2 in citrate buffer, pH 5.0); (ii) biotinylated membrane proteins were detected with HRP-conjugated streptavidin (Chemicon International) and the enzyme substrate solution; (iii) viral antigen was detected by reacting with HRP-conjugated IgG from a flavivirus-specific monoclonal antibody, D1-4G2 (Gentry *et al.*, 1982), and the enzyme substrate solution.

Peaks of HRP-transferrin and biotin activity were detected in fraction nos 4 and 10, respectively (Fig. 2a). The viral antigen was mainly detected in fraction nos 4 to 6, corresponding to the endosome fractions in untreated cells (Fig. 2b). In chlorpromazine-treated cells, the viral antigen was detected in fraction nos 10 to 12, corresponding to the membrane fractions. The results suggest that JEV bound to the cell surface, but did not translocate to the endosomes during the 15 min chase period in chlorpromazine-treated cells. Chlorpromazine did not inhibit the binding of JEV to Vero cells in the concentration range 2.5 to $20 \mu\text{g ml}^{-1}$ (data not shown).

We analysed the effects of chlorpromazine on distribution of the clathrin and viral antigens in the cells by indirect immunofluorescence (Fig. 3). Chlorpromazine-treated and control cells growing on Lab-Tek II chamber slides were inoculated with purified JE virions ($\text{ca } 3.4 \times 10^9$ p.f.u.) in the presence or absence of chlorpromazine at 37°C for 30 min. Cells were washed three times with ice-cold HBSS, and fixed with 90% (v/v) ethanol for 15 min. Then, the cells were reacted with flavivirus-cross-reactive monoclonal antibody D1-4G2 or monoclonal antibody to clathrin heavy chain (Progen). Next the cells were reacted with the FITC-conjugated F(ab')₂ fraction of goat anti-mouse IgG (H+L) (Zymed). Viral antigens were detected as small punctate clusters in the control cells (Fig. 3a) but were absent from the chlorpromazine-treated cells (Fig. 3b). The clathrin heavy chain antigens were detected as a typical punctate pattern in the control cells (Fig. 3c), while only small numbers of faint fluorescent clusters were detected at the perinuclear region in the chlorpromazine-treated cells (Fig. 3d). Fig. 3(e, f) shows JEV antigens detected in the control (e) and chlorpromazine-treated (f) cells on day 4 after infection. The number of antigen-positive cells was less in the drug-treated cells than in the control cells; however,

viral antigens were detected at the same level in both drug-treated and non-drug-treated cells. The results demonstrated that chlorpromazine affected the intracellular distribution of clathrin (Fig. 3c, d), and inhibited uptake of JEV in Vero cells (Fig. 3a, b). However, the presence of chlorpromazine at the early stage of infection did not inhibit the synthesis of progeny viral proteins once infection was established (Fig. 3e, f).

Electron microscopic studies on the entry of flaviviruses demonstrated that virions were taken up into small endocytic vesicles in the cells (Kimura *et al.*, 1986; Ng & Lau, 1988; Ishak, 1988). In other experiments, a group of drugs which elevate pH in intracellular acidic compartments, such as endosomes and lysosomes, was used to determine the site at which the viral genome enters the host cells (Brown *et al.*, 1983). Treatment of a mouse macrophage-like cell line, P388D1, with ammonium chloride inhibited West Nile virus infection (Gollins & Porterfield, 1986). These reports agreed with the report that bafilomycin, a specific inhibitor of vacuolar type H^+ -ATPase, interfered with JEV infection of Vero cells (Nawa, 1997). These reports demonstrated that JEV was taken up by the cells through a constitutive endocytic pathway, and that pH in the vesicles plays an important role in intracellular translocation of the virions. The results in the present study demonstrated that chlorpromazine inhibited the entry process of JEV infection. Subcellular translocation of JEV into the endosome fraction was prevented by the pre-treatment of cells with chlorpromazine. Therefore, although the initial interaction between JEV and Vero cells, including virus binding to unidentified receptor(s), is not understood, the clathrin-dependent endocytic pathway is the subsequent step of entry.

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