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Antiviral Effect of Amphotericin B on Japanese Encephalitis Virus Replication

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Abstract Amphotericin B (AmB), an amphipathic polyene macrolide, is an antifungal drug produced by *Streptomyces nodosus*. Recently, AmB has been shown to exert antiviral activity against rubella virus and human immunodeficiency virus by different mechanisms. In this study, we evaluated the antiviral effect of AmB against Japanese encephalitis virus (JEV) and investigated which step of the viral life cycle was inhibited by AmB to understand the mechanism of antiviral action of AmB. AmB reduced both plaque size and number in the infected cells in a dose-dependent manner. In addition, a 200-fold reduction of infectious virus titer was observed by treatment of infected cells with 5 μg/ml of AmB. AmB acted at the post virus-infection step, but not during adsorption of virus to host cells. Western blot analysis revealed that the accumulated level of JEV envelope protein dramatically decreased in the infected cells by treatment with 5–10 μg/ml of AmB. Our results indicate that AmB inhibits the replication of JEV at the postinfection step by interfering with viral replication and/or by inhibiting the synthesis of viral proteins.

Key words: Japanese encephalitis virus, amphotericin B, antiviral drug, real-time RT-PCR, plaque assay, cytopathic effect

Japanese encephalitis virus (JEV), a member of the *Flaviviridae* family, is a pathogen for an acute viral infection of the central nervous system [3]. The viral genome of JEV is a single-stranded, positive-sense RNA of 11 kb in length and contains one long open reading frame encoding a single polyprotein of approximately 370 kDa, which is co- and/or post-translationally processed into more than 10 viral proteins by cellular and viral protease; the structure proteins including capsid, membrane (M), and envelope (E) proteins, as well as the nonstructural (NS) proteins, NS1 to NS5. JEV replicates in the cytoplasm and buds from the endoplasmic reticulum (ER) or Golgi membrane to release mature virions, which are composed of a lipid bilayer with at least two envelope proteins, M and E, surrounding a nucleocapsid [6, 15]. For the prevention of JEV infection, vaccines using inactivated JEV from infected suckling mouse brain and other cell cultures have been developed [9]. However, no antiviral drugs are yet available for the treatment of infections with JEV.

Streptomyces produce diverse potential antifungal drugs against human and plant pathogens [7, 13]. Amphotericin B (AmB) is a polyene family antifungal drug produced by a strain of *Streptomyces nodosus* [5, 18]. Its has been in clinical use since the early 1960's for the treatment of systemic fungal infections, and has been the mainstay of antifungal therapy for severe systemic mycotic infections despite of its severe side effect, since there have been no better alternatives [1]. As an antifungal drug, AmB binds specifically to ergosterol in the cell membrane of fungi to form an ion channel, and alters potassium and magnesium ion concentration in cells, thereby preventing the propagation of fungi [4, 5]. AmB binds specifically not only to ergosterol in fungi, but also to cholesterol in mammalian cell membrane. It has also been known that interaction of AmB with ergosterol is stronger than with cholesterol even though there are only minor structural differences between ergosterol and cholesterol [8, 10].

Since AmB can possibly act on cholesterol in viral envelopes and membranes of cellular and intracellular organelles, it might have an antiviral effect. Indeed, its antiviral effect has been demonstrated in several enveloped viruses. AmB showed an antiviral effect against rubella virus at a late stage of virus propagation, whereas no antiviral effect against measles and mumps viruses, which belong to *Paramyxoviridae*, was observed [19]. These findings indicate that AmB selectively inhibits the replication of a certain enveloped virus even though rubella, measles, and mumps viruses are all enveloped single-stranded RNA viruses consisting of a membraneous envelope, thus being the potential target of AmB. For human immunodeficiency virus (HIV),

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the antiviral effect of AmB was exerted by binding to cholesterol in the lipid-bilayer of HIV particle envelope. Therefore, AmB was suggested to be useful to block the early steps in HIV entry [12, 14]. Similarly, AmB has an effect on the structural integrity of hepatitis B virus (HBV) particles, aggregation of HBV, and hepatitis B surface antigen, but its antiviral effect has not been demonstrated [11].

Since AmB is expected to alter the structure of the viral envelope, membrane integrity of cells, and internal cellular organelles, we evaluated in the present study the antiviral effect of AmB on replication of JEV causing acute encephalitis by plaque forming assay and analysis of viral RNA levels in the released virions. Decrease of viral protein synthesis was also confirmed by Western blot analysis of JEV envelope protein.

MATERIALS AND METHODS

Virus, Cell Line, and Chemicals

JEV, NIH-Nakayama strain, was obtained from the Department of Viral Disease, Korea National Institute of Health, and used throughout this study. The virus was propagated in baby hamster kidney cells (BHK-21). Cells were grown as a monolayer at 37°C in minimum essential medium (MEM; GIBCO BRL) supplemented with 5% fetal bovine serum (FBS; GIBCO BRL) and 1% penicillin/streptomycin sulfate (GIBCO BRL). Pure form of AmB was obtained from E. R. Squibb & Sons, Inc. A stock solution (5.0 mg/ml) of pure AmB was prepared in phosphate buffered saline (PBS; GIBCO BRL) and stored at - 80°C until used.

Virus Infection and Plaque Forming Assay

For infection with JEV, BHK-21 cells in 6-well plates (Nunc) were adsorbed with JEV for 90 min at 37°C in a 5% CO_2 incubator with gentle shaking every 15 min. After adsorption, unbound viruses were removed by aspiration and gentle washing with serum-free medium. Cells were then overlaid with 3 ml of solid culture medium [5% FBS, 1% penicillin/streptomycin, 1% low melting agarose (SeaPlaque agarose; FMC BioProducts), 1× MEM with 2.9% sodium bicarbonate and 2 mM L-glutamine (GIBCO BRL)]. After incubation for 48 h at 37°C, cells were stained with 1.5 ml of solid culture medium containing 5% neutral red (NR; Sigma). After incubation for 20 h at 37°C, plaque numbers were counted.

Cytopathic Effect on Cells: MTS Assay

The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; Promega] assay was used to measure cell viability. Briefly, MTS was dissolved in Dulbecco's phosphate buffered saline (GIBCO BRL) to make a stock solution (2.0 mg/ml), which was then filtered to remove any insoluble residues. Cells were seeded at a concentration of 2.0×10^3 cells per well of 96-well plates and incubated with various concentrations of AmB for 2 days. The culture medium was changed, and cells were allowed to equilibrate with fresh medium for 1 h, then 30 µl of combined MTS/PMS solution formulated by the manufacturer's instructions was added per well. After 1 h incubation at 37°C, absorbance at 490 nm was measured using an ELISA plate reader (SPECTRA Max 340, Molecular Devices, U.S.A.).

Inhibitory Effect of AmB on Plaque Formation by JEV

BHK-21 cells in 6-well plates were infected with the virus, and plaque forming assays were performed in the presence of various concentrations (0, 1.0, 2.5, 5.0, 10 µg/ml) of AmB added to solid culture medium containing 1.5% low melting agarose. The infected cells were incubated at 37°C for 2 days and then stained with 5% NR added to the secondary agarose overlay medium. At day 1 after secondary overlay, photography was taken to measure the number and size of plaques formed. Similarly, the inhibitory effect of AmB on before, during, or after virus adsorption was tested by treatment of cells with 10 µg/ml AmB for 24 h before JEV infection, for 90 min during adsorption, or for 48 h after adsorption.

Inhibition of JEV Propagation by AmB

BHK-21 cells were infected with JEV at a different multiplicity of infection (MOI) for 90 min at 37°C, washed with PBS, and received MEM containing 2% FBS and various concentrations (1.0, 2.5, 5.0, 10 µg/ml) of AmB. Culture supernatants were harvested at different time points of postinfection, and plaque forming assays were performed. The inhibitory effect of AmB on before, during, or after virus adsorption was also tested by treatment of cells with 1 - 5 µg/ml of AmB for 24 h before JEV infection, for 90 min during adsorption, or for 48 h after adsorption, which was followed by time-course sampling of culture supernatant and plaque forming assay.

Viral RNA Extraction and Real-Time RT-PCR

JEV viral genomic RNA was extracted from culture supernatant using Tri Reagent (Molecular Research Center) by following the manufacturer's instructions. The isolated RNA was suspended in 0.1% diethylpyrocarbonate-treated water. Real-time RT-PCR was carried out as described previously [14] with a slight modification. In brief, amplification was performed with 17.5 µl of Light Cycler RNA amplification SYBR Green I mix containing 2.5 mM $MgCl_2$ (Roche Molecular Biochemicals) and 2.5 µl of RNA template (total RNA prepared from 100 µl culture supernatant) plus 100 pmol each of primers of JE3F1 (5'-CCC TCA GAA CCG TCT CGG AA-3') and JE3R1 (5'-CTA TTC CCA GGT GTC AAT ATG CTG T-3'). PCR was performed in 1

cycle of 10 min at 55°C (reverse transcription), and 40 cycles of 30 sec at 95°C (denaturation), 10 sec at 55°C (annealing), and 13 sec at 72°C (extension), with fluorescence detection at 87°C after each cycle.

Western Blot Analysis

The guinea pig anti-JEV polyclonal antibody (generated in the Department of Viral Disease, Korea National Institute of Health) was used to detect JEV E protein. Cell lysates were resolved on a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane was then blocked in TBS (150 mM NaCl, 25 mM Tris, pH 7.4) containing 5% BSA and reacted overnight at 4°C with anti-JEV polyclonal antibody at a 1:1,000 dilution in TBS blocking solution. The bound antibodies were detected by horseradish peroxidase-conjugated anti-guinea pig immunoglobulin (Sigma) at a 1:10,000 dilution and developed with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech). Monoclonal antibody specific to α-tubulin (Oncogene) was used to verify the equivalent amounts of cell lysates used for immunoblotting.

RESULTS AND DISCUSSION

Cytotoxicity of AmB on BHK-21 Cells

We first investigated whether AmB (Fig. 1) at the dose range of 1.0 to 25 μg/ml shows a cytopathic effect on BHK-21 cells. MTS assay was used to measure mitochondrial function, which served as an index of living cells to indicate noncytotoxic concentrations of AmB on BHK-21 cells. As shown in Fig. 2A, there was no significant difference in cell viability between untreated and treated cells with 10 μg/ml AmB for 2 days, however, a slight reduction of cell viability started to be observed with 20 μg/ml AmB. Figure 2B shows almost no difference in cell morphology between untreated cells and cells treated with 10 μg/ml AmB, indicating no considerable cell damage to BHK-21 cells with up to 10 μg/ml AmB.

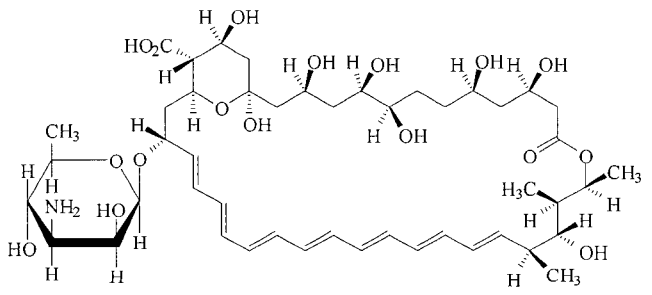

Fig. 1. Structure of AmB.
AmB is an amphipathic polyene antibiotic which permeabilizes ergosterol-containing membranes, probably through forming of pores [4, 5, 18].

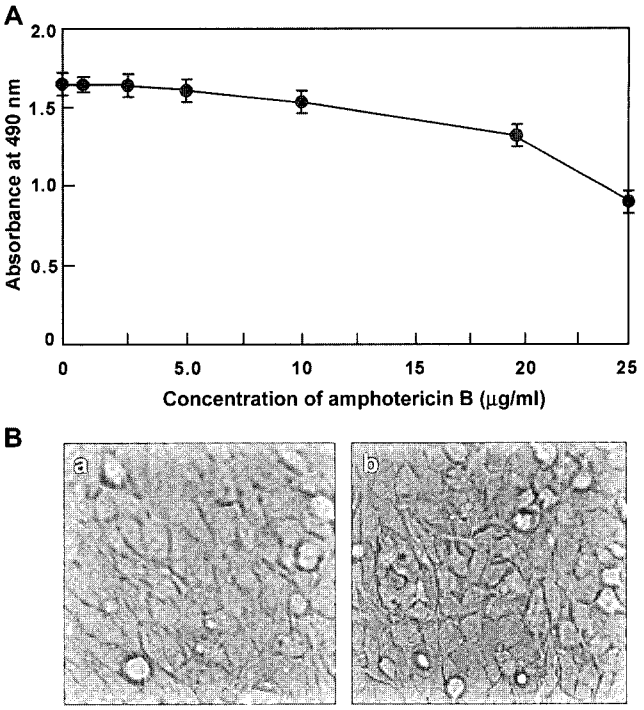

Fig. 2. Cytopathic effect of AmB on BHK-21 cells.
(A) Determination of the subcytotoxic concentration of AmB on BHK-21 cells. Cells were incubated with various concentrations of AmB for 2 days. After 1 h incubation of the AmB-treated cells with MTS/PMS solution at 37°C, absorbance at 490 nm was measured using an ELISA plate reader. Each point represents mean value of triplicates and the vertical bars represent standard error of the mean. (B) Cytotoxicity of AmB on BHK-21 cells. (a) BHK-21 cells as a control. (b) AmB (10 μg/ml)-treated BHK-21 cells. Photographs were taken at 2 days post-treatment of AmB using an inverted microscope.

Inhibitory Effect of AmB on JEV Plaque Formation

Since AmB has an ability to bind the cholesterol in cellular membrane and endomembranes such as lysosome, endosome, endoplasmic reticulum, and Golgi, or possibly on the viral envelope, the antiviral effect of AmB against JEV was investigated first by plaque forming assay. Thus, BHK-21 cells were infected with various dilutions of JEV. After virus adsorption, the cells were washed, and solid culture medium containing various concentrations (0 to 10 μg/ml) of AmB was overlaid. Diameters of plaques were measured at 2 days postinfection after staining of the infected cells by overlaying with secondary agarose medium containing 5% NR. As shown in Fig. 3A, the plaque size was reduced by AmB treatment in a dose-dependent manner. In the presence of 10 μg/ml AmB, the plaque size was reduced to 1/5 of that obtained in the absence of AmB. In addition, plaque formation was inhibited by more than 60%, compared to the plaque numbers (plaque forming unit, pfu) observed on nontreated cells (Fig. 3B). These results indicate that the AmB acted as an antiviral drug against JEV in a dose-dependent manner within the range of concentrations not showing cytotoxicity.

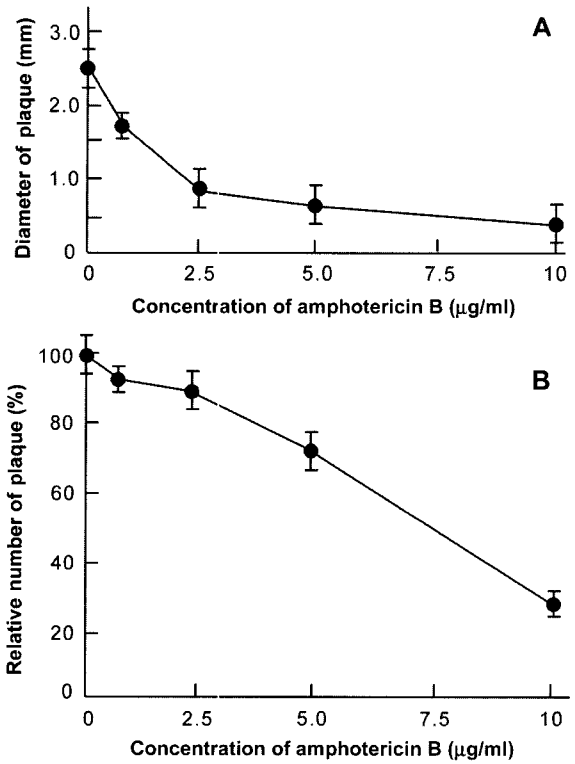

Fig. 3. Inhibitory effect of AmB on plaque formation by JEV.
BHK-21 cells seeded in 6-well plates were infected with JEV. After virus adsorption, the cells were overlaid with solid culture medium containing various concentrations (0 to 10 μg/ml) of AmB. The infected cells were incubated at 37°C and then stained with 5% NR in the secondary agarose overlay medium on day 2. At 1 day after secondary overlay, photography was taken. Data are averages and standard errors from three wells for each concentration. (A) Average diameters of plaques formed by JEV are plotted. (B) Relative numbers of plaques in the cells treated with various concentrations of AmB are plotted.

Inhibition of JEV Propagation by AmB at the Postvirus Adsorption Step

Next, we investigated which step of the virus life cycle was affected by AmB treatment. BHK-21 cells were treated with 10 μg/ml AmB before, during, or after virus adsorption, as outlined in Fig. 4. Cells were infected with appropriately diluted JEV to have countable plaque numbers on each plate, and the plaque formation was observed at 2 days postinfection. As shown in Fig. 4, a significant reduction of viral growth was observed only when AmB was added to the target cells after, but not before or during, virus adsorption. No significant reduction of plaque numbers by treatment of the cells before virus adsorption indicates that AmB does not interfere with virus entry by altering the cellular membrane and/or receptor. Furthermore, lack of antiviral activity by treating both cells and viruses for 90 min during virus adsorption indicated that virion integrity and virus-cell contact were not affected. To quantify the antiviral effect of AmB on each step of the virus life cycle, titers of infectious viruses released from the infected cells

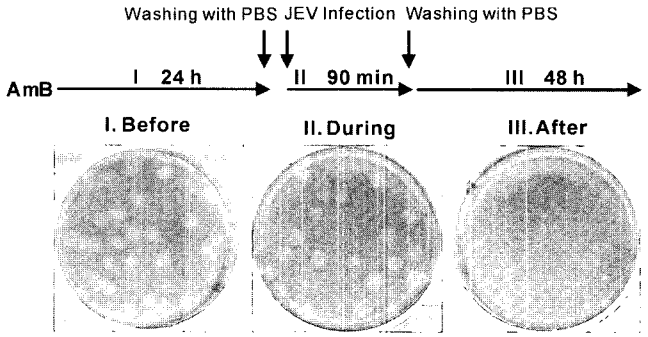

Fig. 4. Antiviral effect of AmB at postvirus adsorption step.
BHK-21 cells in 6-well plates were treated with 10 μg/ml AmB for 24 h before adsorption (I), for 90 min during JEV-adsorption (II), or for 2 days after adsorption (III). After treatment I, cells were infected with JEV. In experiment II, drugs were removed and the cells were incubated further for plaque formation. In experiment III, drugs were left until staining of the cells. Cells were stained with 5% NR at 2 days postinfection. Outline of experimental procedure is shown above the photographs.

were also measured by plaque forming assay at 1 day and 2 days postinfection. No decrease of infectious virion titer was observed until 2 days postinfection, when the cells were treated with 5 μg/ml of AmB before or during virus adsorption (Fig. 5).

In order to investigate whether the antiviral effect of AmB was exerted during viral replication, BHK 21 cells infected with JEV at high MOI (MOI: 5, Fig. 6A) or low MOI (MOI: 0.1, Fig. 6B) were treated with increasing

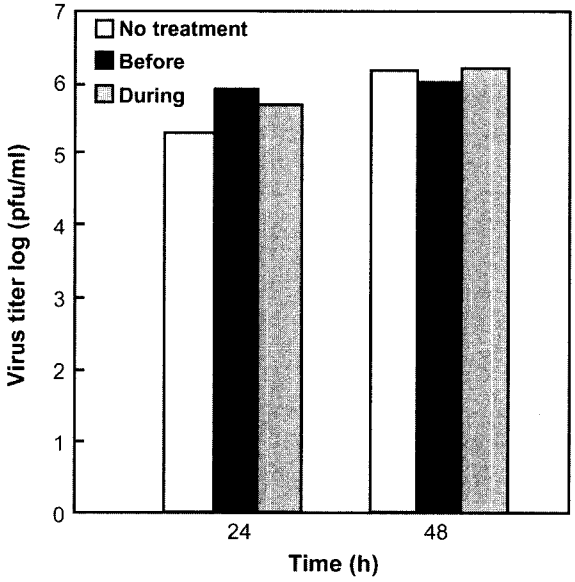

Fig. 5. No antiviral effect of AmB before and during virus adsorption steps.
BHK-21 cells were treated with 5.0 μg/ml AmB before adsorption of JEV for 24 h or during JEV-adsorption for 90 min. After infection of the cells with JEV at an MOI of 5, culture supernatant was harvested for virus titering by plaque forming assay. Means of two independent experiments are shown.

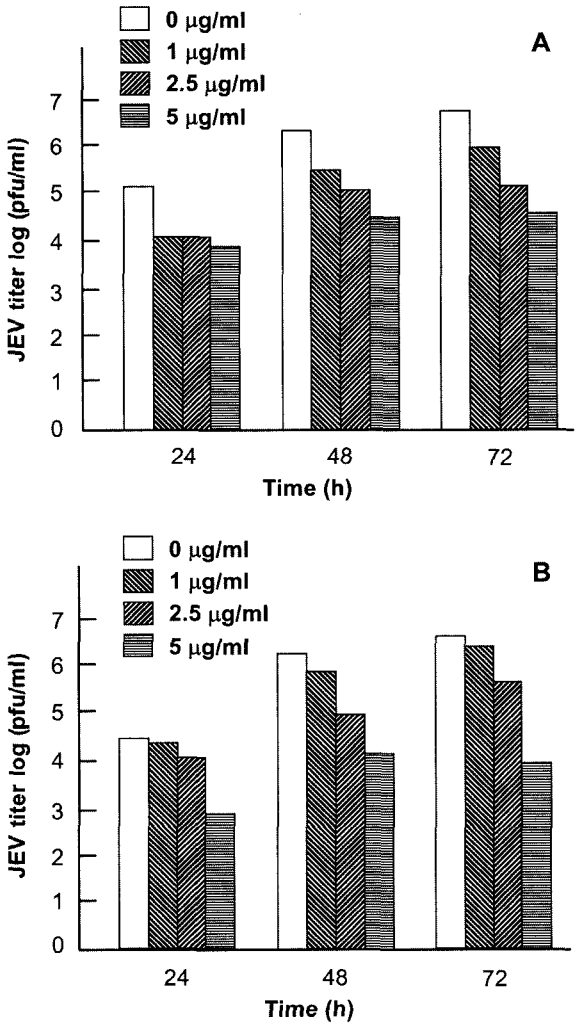

Fig. 6. Inhibition of JEV growth by AmB at postinfection step. BHK-21 cells were seeded at a concentration of 1.0×10^5 cells per wells of 24-well plates and incubated for 2 days at 37°C. After infection with JEV at an MOI of 5.0 (A) or 0.1 (B), culture supernatant was harvested every 24 h for 3 days. Virus titer was measured by plaque forming assay. Means of two independent experiments are shown.

amounts of AmB (1–5 μg/ml). We found that AmB treatment reduced the formation of infectious JEV virions in a does-dependent manner. Titers of infectious virus decreased more than 200-fold by treatment with 5.0 μg/ml AmB at both high and low MOIs of cells. These data indicate that AmB acts mainly on the intracellular step(s) of viral life cycle or during maturation and release of virus rather than on viral entry or directly on the envelope of virus surrounding the viral nucleocapsid, since the incubation of virus with AmB during virus adsorption did not affect the formation of infectious virions. Our results differ from the previous reports with HIV, where an AmB derivative blocked the entry of HIV by binding and altering the envelope of HIV virions [12, 14]. Since JEV mature virions bud from membranes of endoplasmic reticulum (ER) and Golgi apparatus [15] rather than from cellular membrane in the case of HIV [2], it is tempting to speculate that the composition of the JEV viral envelope is different from that of HIV, thus showing a different inhibitory mechanism of AmB on JEV. We further confirmed the inhibitory effect of AmB on JEV replication by quantitative real-time RT-PCR analysis of viral RNA genome in the released virions. The real-time RT-PCR could detect JEV viral RNA derived from as low as 10 pfu of virus. BHK-21 cells infected with JEV at an MOI of 5 and treated with 5.0 μg/ml AmB for 2 days yielded approximately a 100-fold reduced amount of viral genome (data not shown), which is consistent with the result obtained by plaque forming assay, as shown in Fig. 6A. This result also supports the finding that inhibitory effect of AmB against JEV is not due to the inactivation of virion infectivity by acting directly on JEV virions during virus adsorption as shown in Fig. 4, since both amount of viral genome and infectious viral titer were decreased similarly.

Kinetics of the Inhibition of JEV Replication by AmB

To further evaluate the antiviral effect of AmB on the cells infected with JEV at high MOI, we measured the titers of infectious virions released from the BHK-21 cells infected with JEV at an MOI of 10 and treated with 5.0 μg/ml AmB at different postinfection time points. Reduction of virus titer in the supernatant started to be observed at the early stage of virus infection (6 h postinfection) and was more prominent from 12 h postinfection. The virus titer was reduced by approximately 200-fold at 2 days postinfection

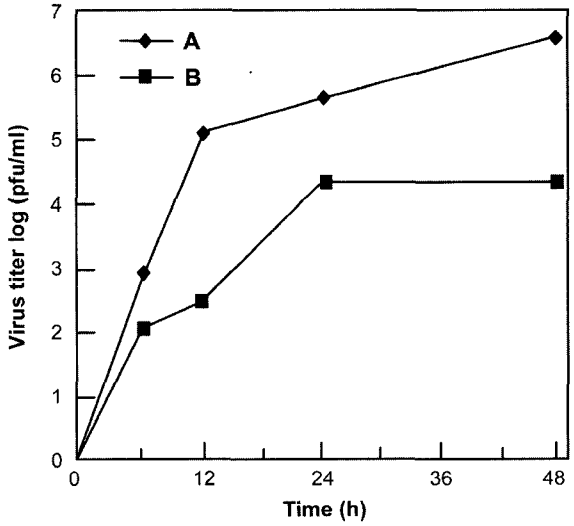

Fig. 7. Kinetics of JEV growth inhibition by AmB. BHK-21 cells were seeded at a concentration of 1.0×10^5 cells per well of 24-well plates and incubated at 37°C for 2 days. After infection (MOI of 10), culture supernatant was harvested at different time points for 2 days. Virus titer was measured by plaque forming assay. Means of two independent experiments are shown. A, Nontreated; B, 5.0 μg/ml of AmB-treated.

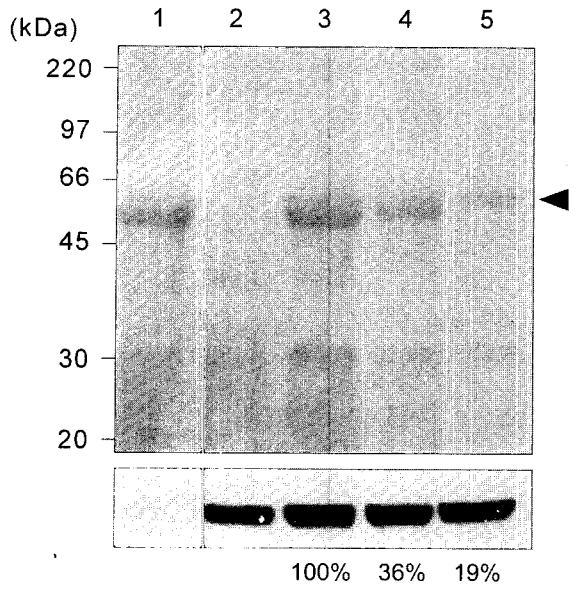

Fig. 8. Inhibition of JEV envelope protein synthesis by AmB. Crude cell lysates from BHK-21 cells mock-infected (lane 2), infected with JEV at an MOI of 5.0 (lane 3), or infected with JEV and treated with 5.0 μg/ml (lane 4) or 10 μg/ml (lane 5) AmB were analyzed at 48 h postinfection. Cell lysates and purified JEV virions (lane 1) as a control were separated by 12% SDS-PAGE and probed with anti-JEV antibody (top panel). Equal amounts of lysates were confirmed by anti-α-tubulin antibody (bottom panel). Positions of protein molecular weight markers are indicated on right. Arrowhead indicates the position of JEV E protein. Densities of the E protein bands measured by densitometery and normalized by using the respective tubulin protein bands are shown below the bottom blot as percentages of the control level (lane 3).

(Fig. 7). This result is consistent with the data obtained with relatively lower MOI, as shown in Fig. 6.

Inhibition of JEV Envelope Protein Synthesis by AmB

The influence of AmB on the expression of viral envelope protein (E protein) was examined by Western blot analysis. BHK-21 cells were mock infected (Fig. 8A, lane 2), infected with JEV at an MOI of 5 (lane 3), or infected and treated with 5 or 10 μg/ml AmB (lanes 4-5) for 2 days. Cell lysates from those cells and purified JEV virions were analyzed for the detection of JEV E protein (about 50 kDa) by Western blot analysis with anti-JEV antibody. In the presence of 5.0 and 10 μg/ml AmB, the accumulated level of JEV E protein was reduced by 64% and 81%, respectively, indicating that AmB affected the synthesis of viral proteins directly or indirectly by inhibiting the replication of viral RNA. Recently, it has been reported that endoplasmic reticulum stress and unfolded protein response are induced by JEV infection [17]. AmB might interfere with synthesis and/or maturation of viral glycoproteins (envelope, prM, NS1) within the endoplasmic reticulum (ER) lumen by impairing the function of ER, since the binding of AmB to the cholesterol in ER membrane would make ER more susceptible to damage upon viral infection. Alternatively, AmB might change the microenvironment on ER, the JEV replication site [15], thus resulting in the inhibition of viral RNA replicase complex formation on ER.

Although the antiviral effect of AmB against JEV still needs to be evaluated *in vivo*, targeting of drugs on membraneous structures of eukaryotic host cells and the viral envelope might be an important strategy for the development of a novel antiviral drug.

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