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Effect of lipopolysaccharide pre-treatment on the replication of Japanese encephalitis virus in microglia

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Abstract

Microglia are resident cells of the central nervous system (CNS) and become activated to function as brain macrophage in response to infectious pathogens. Our recent findings further demonstrate that microglia support the replication of Japanese encephalitis virus (JEV) and remain productively infected for up to 16 weeks. Thus, microglia may serve as a viral reservoir for JEV infection of neurons in the CNS in addition to provide the first line of defense against invading pathogens. Interestingly, in the same study, no increase in nitric oxide levels in the supernatant of microglial cultures was observed following JEV infection over a 5 day period. This suggests that microglia may not be activated in response to JEV infection. Therefore, using the same experimental paradigm this study aimed to determine whether activation of microglia prior to JEV infection would reduce a subsequent viral production. Briefly, mouse BV-2 microglia were plated at a density of 5×10^5 cells/well in 6-well plates. Then, the cultures were treated with lipopolysaccharide (LPS) prior to the infection with JEV at a MOI of 5 pfu/cell. At 24 h post-infection, the growth media containing progeny virus were collected for determination of viral titer by standard plaque assay. Results clearly demonstrated that LPS pre-treatment dramatically reduced viral titer in the growth media compared with that of infected cultures without LPS pre-treatment. Thus, cellular activation by LPS prior to the infection appears to reinforce a natural innate immune mechanism of microglia against JEV infection.

Keywords: Japanese encephalitis virus, microglia, LPS

Introduction

Japanese encephalitis virus (JEV) is a member of the family *Flaviviridae*, which includes West Nile, Dengue, Tick-borne encephalitis and Yellow fever viruses. The JEV genome consists of a positive single-stranded RNA approximately 11 kb in length [1]. JEV replicates exclusively in the cytoplasm and mature on intracellular membranes, but not on plasma membrane of infected host cells [2]. JEV is the most common cause of human arthropod-borne encephalitis in Asia with a high mortality rate. The clinical manifestation of JEV infection including fever, headache, vomiting and altered consciousness leading to neurological sequelae in some of those who survive [3]. In infected human cases, JEV antigens were localized mainly in neurons suggesting that neurons are the principal target cells of JEV [4]. However, recent study have demonstrated that microglia support the replication of JEV and remain productively infected for up to 16 weeks [5]. Thus, microglia may serve as a viral reservoir for JEV infection of neurons in the central nervous system (CNS).

Microglia are the resident immune cells of the CNS and have a critical role in host defense against invading pathogens [6]. Activation of microglia *in vitro* can be induced by a wide range of stimuli, including lipopolysaccharide (LPS) which is outer membrane glycolipids of gram-negative bacteria. LPS activates microglia through toll-like receptor 4 (TLR4) resulting in the production of proinflammatory cytokines, chemokines and reactive

oxygen/nitrogen species [7]. Specifically, Nitric oxide (NO) and interferons (IFNs) secreted by activated microglia play a critical role in antimicrobial action and inhibition of viral replication in the CNS [6, 8]. However, our previous results demonstrated that infection of microglia with JEV did not induce NO production over a 5 day period [5]. Thus, we hypothesize that an absence or a delay of microglial activation following JEV infection may be beneficial to the replication of JEV. To test this hypothesis, cultures of microglia were infected with JEV with or without LPS pre-treatment. At 24 h post-infection, the growth media containing progeny virus were collected for determination of viral titer by standard plaque assay as described in the methods section.

Materials and methods

Cell cultures, C6/36 cells were cultured in minimum essential medium (MEM) supplemented with 10% FBS, 1% L-Glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin and used to propagate JEV. LLC-MK2 and BV-2 cells were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 5-10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% L-Glutamine at 37°C in a 5% CO_2 incubator. All cell culture reagents were purchased from Hyclone (USA).

JEV propagation, C6/36 cells were grown in tissue culture flasks until sub-confluence. As previously described [5], the culture medium was replaced with serum-free MEM containing the JEV strain Beijing-1 (accession No.L48961) at multiplicity of infection of 1 pfu/cell. Viral infection was allowed to proceed for 120 min at 28°C with constant agitation. Subsequently, fresh complete MEM was added to the cells. At day 4 post-infection, the medium containing progeny virus was collected for determination of viral titer by standard plaque assay.

JEV infection of microglial cells, BV-2 microglia were plated at a density of 5×10^5 cells/well in 6-well plates and incubated with 0.5 μg/ml of LPS (Sigma, USA) for 6 h at 37°C. Untreated cultures were served as control. Then, the cells were washed with PBS 3 times and subsequently inoculated with JEV at a MOI of 5 pfu/cell for 2 h at 37°C followed by washing with PBS to remove unabsorbed viruses. Uninternalized extracellular viruses were inactivated by washing with acid glycine buffer followed by washing with PBS. Then, fresh serum-containing medium was added into the cells and the cells were incubated at 37°C in a 5% CO_2 incubator. The media containing progeny virus were collected at 24 h post-infection for determination of viral titer by standard plaque assay.

Standard plaque assay, To determine viral titer, LLC-MK2 cells in 6-well plates were inoculated with 200 μl of serially diluted virus-containing medium for 1 h and 30 min at 37°C with agitation. Then, 1% Seakem LE agarose (Cambrex, USA) mixed with nutrient overlay (Earle's Balanced Salts supplemented with 0.5% (w/v) yeast extract, 2.5% lactalbumin hydrolysate, 3% FBS) was added to each well. The plates were incubated at 37°C in a 5% CO_2 incubator for 7 days. Then, the cultures were fixed with 3.7% formaldehyde and agarose plugs were removed. Plaques were counted after staining with 1% crystal violet solution.

Statistical analysis, All data were presented as mean ± SEM. One way analysis of variance followed by LSD was used to compare the significance between the control and the treatment groups. The p-value of less than 0.05 was set for the significant difference.

Results

BV-2 cells were infected with JEV at a MOI of 5 pfu/cell at 37°C for 2 h. At 24 h post-infection, the growth medium was collected and serially diluted to quantify progeny viruses by standard plaque assay (Fig. 1 A). The calculated viral titer was $5\pm0.5\times10^5$ pfu/ml (Fig. 1 B). No plaques were detected in uninfected control cultures.

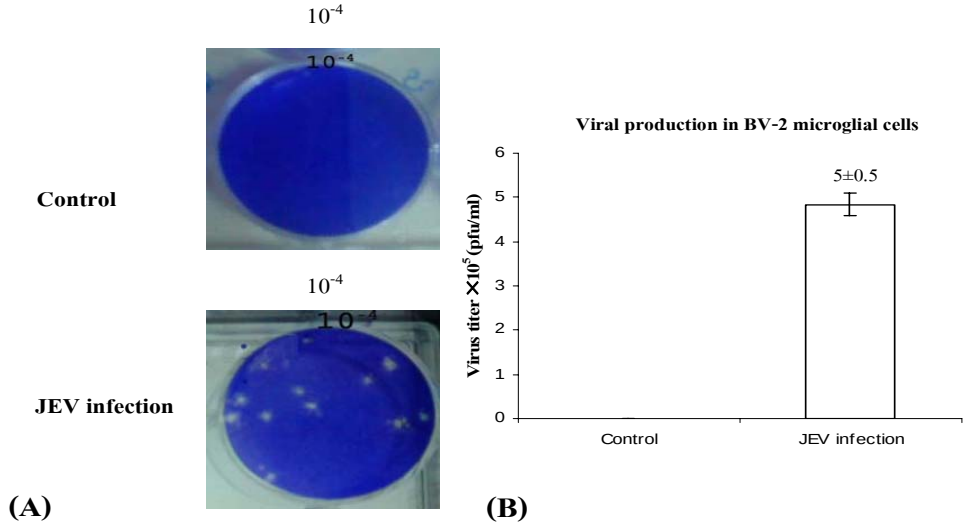

Figure 1. JEV infection of microglia. BV-2 cells were infected with JEV at a MOI of 5 pfu/cell. Virus-containing growth medium was collected at 24 h post-infection, serially diluted and used to inoculate LLC-MK2 cells, according to a standard plaque assay procedure. This experiment was repeated three times independently and each was performed in triplicate. (A) Plaques were stained with 1% solution of crystal violet. (B) The viral titers are displayed graphically.

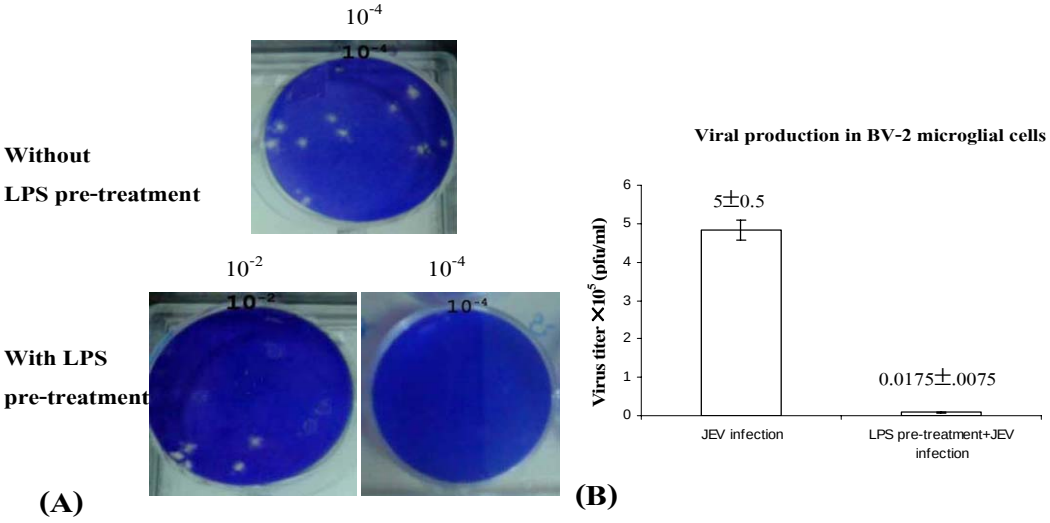

Figure 2. JEV infection of microglia with or without LPS pre-treatment. BV-2 cells were pre-treated with LPS (0.5 μg/ml) before JEV infection (5 pfu/cell). Virus-containing growth medium was collected at 24 h post-infection, serially diluted and used to inoculate LLC-MK2 cells. This experiment was repeated three times independently and each was performed in triplicate. (A) Plaques were stained with 1% solution of crystal violet. (B) The viral titers are displayed graphically.

To test whether activation of microglia prior to JEV infection reduces the amount of progeny viruses in the growth medium, BV-2 cells were pre-treated with LPS for 6 h followed by challenge with JEV at MOI of 5 pfu/cell. At 24 h post-infection, progeny viruses in the growth medium were quantified by standard plaque assay (Fig. 2 A). The calculated viral titer in the infected cultures without LPS pre-treatment was $5\pm0.5 \times 10^5$ pfu/ml, whereas

that of infected cultures with LPS pre-treatment was $1.75\pm0.95 \times 10^3$ pfu/ml (Fig. 2 B). The results clearly demonstrated that LPS pre-treatment dramatically reduced viral titer in the growth media compared with that of infected cultures without LPS pre-treatment.

Discussion

Since it was shown that replication of JEV in microglia did not induce the production of NO, a marker associated with microglial activation, an absence or a delay of microglial activation following JEV infection may be beneficial to the replication of JEV. To test this hypothesis, cultures of microglia were infected with JEV with or without LPS pre-treatment. At 24 h post-infection, the growth media containing progeny virus were collected for determination of viral titer by standard plaque assay as described in the methods section. In this experimental paradigm, microlgial activation by LPS occurs through TLR4 which is linked to a variety of intracellular signaling cascades leading to transcription activation of genes involved in the innate immune response. These genes include inducible nitric oxide synthase (iNOS) and interferons (IFNs) [7]. This information strongly supports the present finding that LPS pre-treatment dramatically reduced the production of infectious progeny of JEV from infected microglia. Additional supportive evidence include a recent report by Boivin et al [8] showing that pre-treatment with TLR antagonists induced early expression of several immune genes in the brain and resulted in a significantly lower viral load in a mouse model of Herpes Simplex Virus Type 1 Encephalitis. In summary, the present study demonstrated that cellular activation through TLR4 prior to the infection appears to reinforce a natural innate immune mechanism of microglia against JEV infection.

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