

Method

Bacterial Cell Surface Display: a Method for Studying Japanese Encephalitis Virus Pathogenicity

Jianlin Dou^{1,2,3}, Janet Daly^{2*}, Zhiming Yuan¹, Tao Jing³, and Tom Solomon²

¹State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan;

³Institute of Pathogenic Biology, School of Basic Medical Sciences, Lanzhou University, Lanzhou, China; and

²Viral Brain Infections Group, Divisions of Neurological Science, and Medical Microbiology, School of Tropical Medicine, University of Liverpool, Liverpool, England

(Received April 8, 2009. Accepted July 27, 2009)

SUMMARY: Infection with Japanese encephalitis virus (JEV), a mosquito-borne, neurotropic flavivirus, may cause acute encephalitis in humans. Recombinant *Salmonella typhimurium* BRD509 was constructed to display domain III of the envelope (E) protein of JEV (JEDIII) on its surface with the N-terminal domain of ice nucleation protein (INPN) as the display motif. Bacterial cell surface display was confirmed by Western blot analysis and immunohistochemical staining. Binding of recombinant INPN-JEDIII and JEDIII proteins to three mammalian cell lines was compared using a cell-binding ELISA; the human neuroblastoma cell line SK-N-SH, which had a low level of binding, was selected for further studies. The display of JEDIII on the surface of BRD509 did not significantly influence its invasiveness was confirmed by measuring released bacterial antigen using whole-cell ELISA. The relative expression of an apoptosis-related gene and total DNA damage were assessed to investigate the effects of infection on SK-N-SH cells. Compared to BRD509, infection with the recombinant bacterium reduced cell damage, suggesting that JEDIII may limit apoptosis during the early stages of JEV infection. Our studies demonstrated that it is feasible to study the pathogenesis of JEV using the approach described.

INTRODUCTION

Japanese encephalitis (JE) is the most important epidemic encephalitis, with 30,000 to 50,000 reported cases and more than 10,000 deaths annually (1). It occurs mainly in Asia, but it is gradually spreading to other regions (2); prevention and control of JE is therefore becoming a worldwide problem. JE is caused by an arthropod-borne member of the genus *Flavivirus* (family *Flaviviridae*), Japanese encephalitis virus (JEV). It is a small, enveloped virus with a single-stranded positive sense RNA genome, approximately 11 kb long, encoding three structural proteins including envelope (E) protein, pre-membrane (prM) protein and capsid (C) protein, and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) (3). E protein, exposed on the surface of the virion particle, is regarded as the receptor-binding protein, and is known to contain many epitopes (4,5). It can be structurally divided into three domains: I, II, and III (6,7).

The mechanisms by which JEV causes encephalitis in people are not clearly understood. It is known that JEV is neurotropic, and neuronal apoptosis is regarded as one of the main reasons for neural cell damage. It is well known that viruses play dual roles with respect to apoptosis: blocking it or increasing it. At the early stage of virus infection, apoptosis is blocked to protect the host cell in order to minimize premature virion release; at the late stage, apoptosis can be increased to maximize mature virion release from lytic cells. Most studies have focused on how viruses stimulate apoptosis; however, their blocking effects on apoptosis are poorly

understood. If some viral components have inhibiting effects on apoptosis, they have potential as a subunit vaccine. Unfortunately, it is difficult to separate the influence of different components when apoptosis is ongoing, and new methods to study apoptosis are urgently required.

It was reported that *Salmonella typhimurium* expressing a dengue virus (DENV) epitope on its surface can be used to study immunity to DENV (8). This suggests that it is possible to simulate a viral infection by allowing bacteria displaying viral proteins to infect host cells. *S. typhimurium* is a Gram-negative, facultative intracellular bacterium. Due to its ubiquitous ability to invade eukaryotes, *S. typhimurium* can be used to transport plasmids into host cells (9). *S. typhimurium* BRD509 is an attenuated *aroA*, *aroD* mutant derived from *S. typhimurium* SL1344. Plasmids derived from pBR322 are quite stable in this strain (10).

Ice nucleation protein (INP) from *Pseudomonas syringae* can be attached to the surface of Gram-negative bacteria (11). INP contains three domains: an N-terminal unique region composed of 191 amino acids, a central repeating domain (CRD), and a C-terminal unique region composed of 49 amino acids. The N-terminal domain can be anchored to the membrane via glycosylphosphatidylinositol, whereas the specific C-terminal region (INPC) is highly hydrophilic and exposed to the outermost cell surface. The CRD consists of repeated amino acid sequences with lengths of 8-, 16-, and 48- residues which act as templates for ice crystal formation. Foreign proteins can be inserted in the CRD or fused with the N-terminal domain with removal of the CRD and C-terminal domains (INPN). The fusion of the N- and C-terminal domains without CRD (INPNC) also can be used to display foreign proteins. Large proteins can be displayed using INPN (12,13), but the addition of the C-terminal domain of INP may improve the stability of the fusion protein (14).

In this study, we constructed recombinant *S. typhimurium*

*Corresponding author: Mailing address: Brain Infections Group (Professor Tom Solomon), University of Liverpool, 8th Floor, Duncan Building, Daulby Street, Liverpool, L69 3GA, UK. Tel: +44 151 706 4603, Fax: +44 151 706 5805, E-mail: janet.daly@me.com

BRD509 expressing a JEDIII fusion protein on its surface and used it to infect mammalian cells. We further assessed its effects on SK-N-SH cells and found this to be a practical approach to study the pathogenesis of JEV.

MATERIALS AND METHODS

Bacterial strains and mammalian cell lines: *Escherichia coli* TOP10 (a kind gift from Lin Li, Huazhong Agricultural University, China) was used as the bacterial host for plasmid construction and protein production. *S. typhimurium* BRD509 (a kind gift from Derek Pickard, the Wellcome Trust Sanger Institute, Cambridge, UK) was used as a host for the surface display of JEDIII. Both bacterial strains were cultured in Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) at 37°C or 25°C, and 100 µg/ml ampicillin was added for culture of bacteria containing plasmid.

Cells of the human neuroblastoma cell line SK-N-SH (obtained from the European Collection of Cell Cultures) were grown in Dulbecco's minimal essential medium (DMEM; GIBCO®, Invitrogen Ltd., Paisley, UK) supplemented with 2 mM glutamine and 10% fetal calf serum at 37°C with 5% CO₂. African green monkey kidney (Vero) cells and baby hamster kidney (BHK) cells were grown in RPMI 1640 medium (Gibco) supplemented with 5% fetal calf serum at 37°C in a humidified atmosphere with 5% CO₂. All cell culture media were also supplemented with 100 µg/ml penicillin and 100 µg/ml streptomycin.

Plasmid construction: Plasmid pMPL003 (also a kind gift from Lin Li) containing the full-length gene *inaK* of *P. syringae* (KCTC1832) was used as a template to amplify a 573-bp fragment encoding the N-terminal of INP using the primers InpN-F and InpN-R (Table 1). Plasmid pTINPN was constructed by inserting the *Xho*I and *Pst*I digested PCR product into pTrcHisC (Invitrogen) digested with the same enzymes. The 333-bp PCR product encoding JEDIII (residues 292-402 of JEV E protein) was amplified from pCJEVPrME (unpublished) containing full-length prM and E genes of JEV (Beijing P3 strain) using primers JEDIII-F and JEDIII-R (Table 1). The JEDIII fragment was *Xho*I and *Kpn*I digested and inserted into pTrcHisC to obtain pTrcJEDIII. Binary hybrid pTIJEDIII, INPN fused with JEDIII, was constructed by inserting the 573-bp fragment cut from pTINPN using *Xho*I and *Pst*I into pTrcJEDIII. Plasmid construction is illustrated in Fig. 1. Recombinant plasmids were electroporated into TOP10 and *S. typhimurium* BRD509 cells and confirmed by sequencing. Recombinant TOP10 and *S. typhimurium* BRD509 bearing pTIJEDIII were designated as T-pTIJEDIII and B-pTIJEDIII, respectively.

Cell fractionation: Cell fractionation was performed according to previously described methods (14,15). Briefly, B-pTIJEDIII or T-pTIJEDIII was cultured at 250 rpm at 37°C to reach an optical density at 600 nm (OD_{600nm}) of 0.4 prior to induction with a final concentration of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich, St. Louis,

Mo., USA), and was then cultured at 25°C for 24 h. Cells harvested by centrifugation were washed with phosphate buffered saline (PBS, 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na₂HPO₄, 0.24 g/L KH₂PO₄, pH 7.4) supplemented with 0.5 mM EDTA and resuspended in the same buffer supplemented with protease cocktails (200 mM phenylmethanesulphonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml pepstatin A and 1 µg/ml aprotinin, Sigma-Aldrich). Cells were then lysed by ultrasound sonication (30 s × 6 cycles) on ice. After the removal of cell debris and unlysed cells by centrifugation at 3,000 g for 15 min at 4°C, the supernatant was pelleted by centrifugation at 39,000 rpm for 1 h at 4°C using an ultracentrifuge (Optima LE-80K; Beckman Coulter Inc., Fullerton, Calif., USA). The harvested pellet was regarded as the total membrane fraction, and the remaining supernatant was regarded as the soluble cytoplasmic fraction (CP). The total membrane fraction pellet was resuspended with PBS buffer containing 0.01 mM MgCl₂ and 2% Triton X-100 and incubated at room temperature for 30 min to solubilize the inner membrane fraction (IM). The pellet harvested after further ultracentrifugation (as above) was regarded as the outer membrane fraction (OM). Fractionated samples were analyzed by Western blot.

Protein purification: TOP10 cells bearing pTrcJEDIII or T-pTIJEDIII were grown overnight at 37°C in LB-medium

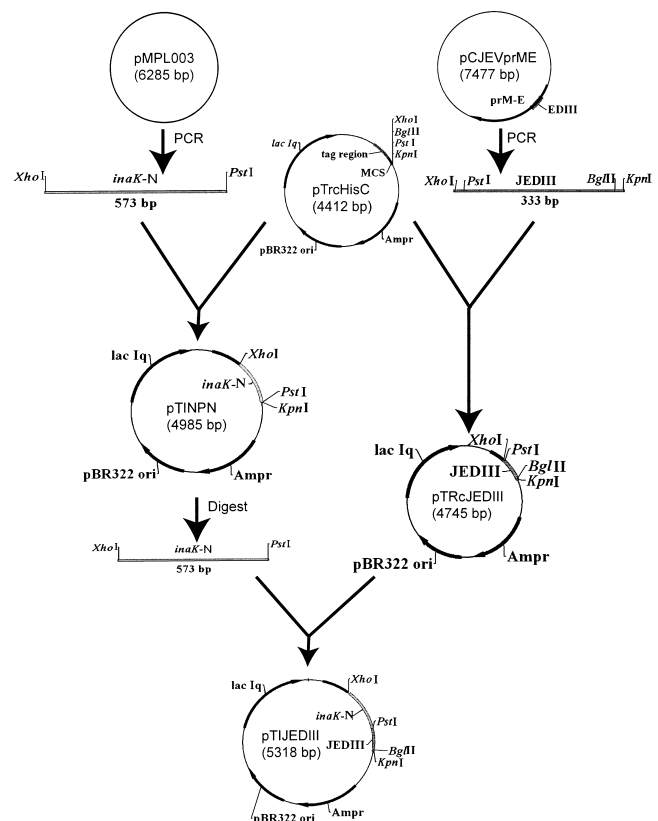


Fig. 1. Schematic diagram of construction of plasmids pTrcJEDIII, pTINPN, and pTIJEDIII.

Table 1. Primers used for plasmid construction

Primer	Sequence 5'-3'
INPN-F	ggg ctcgag ccatgactctcgacaaggcgttgg
INPN-R	ggg ctcgag gtgattgtcgccactcaacgtg
JEDIII-F	ccc ctcgag aaatg ctgcag gacaaactggctctgaaaggcaca
JEDIII-R	ccc ggtacc attaaa gatcct tcgtgcttccagctttgtgccaatgg

Restriction enzyme sites are in bold typeface.

with ampicillin (100 $\mu\text{g/ml}$). Cultures were then diluted to 1:100 in fresh medium and grown to an $\text{OD}_{600\text{nm}}$ of 0.4 at 37°C at a shaking speed of 250 rpm. The expression of recombinant JEDIII protein was induced by adding IPTG to a final concentration of 1 mM for 3 h at 37°C. The expression of fusion protein INPN-JEDIII was induced with the same concentration of IPTG for 24 h at 25°C. Bacterial cells were harvested at 3,000 g for 15 min by centrifugation from 50-ml cultures of TOP10 bearing pTrcJEDIII or 500 ml cultures of T-pTIJEDIII. The latter was used to separate the OM first. Pellets of whole bacterial cell or OM were used to purify protein using a Ni-NTA purification system (Invitrogen) at denaturing conditions according to the manufacturer's instructions. The content of purified protein was measured using the BCA Protein Assay Kit (Pierce, Rockford, Ill., USA) with bovine serum albumin (BSA) as the standard. Purified protein was analyzed by Western blot and SDS-PAGE.

Western blot analysis: Fractionated samples (OM, IM and CP) of the cells were mixed with 5 \times SDS sample buffer (10% sodium dodecyl sulfate [SDS], 100 mM dithiothreitol, 0.3 M Tris-HCl, pH 6.8, 0.05% bromophenol blue, 50% glycerol), boiled for 5 min, and resolved by 12% (wt/vol) SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrophoretic transfer to Hybond-PVDF membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) with a transfer buffer (48 mM Tris-HCl, 39 mM glycine, 20% methanol, pH 9.2) using a Bio-Rad Mini Cell (Bio-Rad, Hercules, Calif., USA) at 250 mA for 2 h. After blocking for 1.5 h in PBS buffer containing 3% (wt/vol) BSA, the membrane was then incubated for 1.5 h at room temperature in PBS-T (PBS with 0.1% Tween-20) supplemented with 0.1% (wt/vol) BSA containing mouse monoclonal antibody Anti-Xpress (1:5,000; Invitrogen). The membrane was washed twice with PBS-T for 10 min, and probed with secondary anti-mouse IgG conjugated with peroxidase (1:1,000 vol/vol; Sigma-Aldrich). After three 10-min washes with PBS-T, One-Step™ TMB-Blotting Substrate (KPL, Gaithersburg, Md., USA) was added and color development was quenched with distilled water after 10 min at room temperature. Purified protein INPN-JEDIII and JEDIII were also analyzed by Western blot using polyclonal rabbit serum against JEDIII as the detecting antibody and anti-rabbit IgG conjugated with peroxidase (1:1,000 vol/vol; Sigma-Aldrich) as a secondary antibody. The membranes were photographed and analyzed using the GeneSnap software provided with the Chemigenius Bioimaging System (Syngene, Cambridge, UK).

Cell-binding ELISA: Confluent monolayers of BHK, Vero, and SK-N-SH cells in 96-well plates were rinsed with PBS and then fixed by adding 10% formaldehyde (Sigma-Aldrich) overnight at 4°C prior to blocking with 3% BSA in PBS. Recombinant protein at high, medium, and low concentrations (440, 220, and 110 ng/well for INPN-JEDIII and 200, 100, and 50 ng/well for JED III, respectively, because the molecular weight of INPN-JEDIII is around 2.2-fold that of JEDIII) was incubated with fixed cell monolayers for 1.5 h at room temperature. After being washed with PBS, the wells were incubated with polyclonal rabbit serum against JEDIII. The bound antibodies were detected after incubation with the anti-rabbit IgG conjugated to peroxidase (KPL) for 1.5 h at room temperature. The ELISA products were developed with SureBlue TMB Microwell Peroxidase Substrate (KPL), the absorbance at 450 nm (A450) was then measured (16), and the A450 of control cells (no protein added) was subtracted.

Invasion assay: SK-N-SH cells were inoculated in 6-well

or 96-well Corning plates or 25-ml flasks. *S. typhimurium* BRD509 and B-pTIJEDIII for invasion assays were prepared according to the procedure as mentioned above. Bacterial cultures were replaced with PBS in mock infections. SK-N-SH cells were infected at a multiplicity of infection of 20:1 in 6-well plates and 25-ml flasks or 70:1 in 96-well plates for 2 h at 37°C in the presence of 5% CO₂. After 2 h incubation, cells were washed three times with PBS containing 100 $\mu\text{g/ml}$ gentamicin to remove extracellular bacteria. When 6 h infection was required, cells were cultured with fresh DMEM supplemented with 100 $\mu\text{g/ml}$ gentamicin after washing. The cells cultured in flasks were used to isolate mRNA, cells cultured in 6-well plates were used to extract total DNA, and cells cultured in 96-well plates were used in whole-cell ELISA.

Whole-cell ELISA: To assess the invasion frequency of bacteria after induction, bacteria were added to SK-N-SH cells in 10 wells of a 96-well plate (9 test wells and control well 1). No bacteria were added to two wells (control wells 2 and 3). After 2 h incubation, all wells were washed six times with sterilized PBS, then 50 μl of sterilized dH₂O was added to the test wells to lyse cells to release intracellular bacteria and to control well 3; cells in control wells 1 and 2 were fixed using 4% paraformaldehyde. After the cells were completely lysed, 50 μl of 2 \times PBS was added to the test wells and control well 3. The plate was incubated at 4°C overnight. Then, the 96-well plate was incubated at 60°C for 30 min, followed by two washes with PBS. Wells were blocked with 200 μl of PBS containing 3% (wt/vol) BSA for 1.5 h at 37°C. All subsequent steps were carried out at room temperature. The wash procedures for control wells 1 and 2 were performed according to the method mentioned in section 2.6 (cell-binding ELISA); none of the buffer solutions contained Tween 20. After the blocking solution was removed, the wells were incubated for 1.5 h with rabbit anti-O4 serum (Remel Europe Ltd., Dartford, UK) at a dilution of 1:3,000. After another extensive washing, the cell-antibody complex was incubated for 1.5 h with goat anti-rabbit IgG-peroxidase conjugate at a final dilution of 1:5,000. Finally, the wells were washed five times with PBS-T, and Sure-Blue TMB was added as a substrate. After the addition of 50 μl of 2 N H₂SO₄, the absorbance of each well was measured at 450 nm. The absorbance of control well 3 was used as zero, and the subtraction of the A450 of control well 2 from that of control well 1 was regarded as indicating the amount of extracellular *Salmonella* antigen remaining after washing; therefore, the amount of intracellular *Salmonella* antigen was calculated by subtracting this value from the A450 of the test wells.

Immunohistochemical staining: Recombinant strain B-pTIJEDIII and *S. typhimurium* BRD509 (as a negative control) were induced with 0.1 mM IPTG, cultured at 25°C for 24 h and harvested by centrifugation. The harvested cells were washed four times with TBS buffer (20 mM Tris-Cl, 500 mM NaCl, pH 7.5). Cells were blocked at room temperature for 2 h in TBS buffer containing 3% BSA, and then incubated in the solution of anti-Xpress (1:5,000 vol:vol) containing 0.1% BSA at room temperature for 1.5 h. After being washed three times in TBS, the cells were incubated with anti-mouse IgG conjugated alkaline phosphatase solution (1:5,000 vol:vol) for 2 h. After another five washes with TBS, the cells were incubated in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl₂) containing nitroblue tetrazolium (Sigma-Aldrich) and 5-bromo-4-chloro-3-indolylphosphate (Sigma-Aldrich) for 30 min to let the col-

Table 2. Primers for semi-quantitative RT-PCR

Primer	Sequence 5'-3'	Expected size (bp)	Reference
TRADD-F	cgctctgtgggtctcaaatggc	248	George et al., 2007 (18)
TRADD-R	agtcctctgccaggctggtag		
β -actin-F	gctcgtcgtcgacaacggctc	353	Invitrogen
β -actin-R	caaacatgatctgggtcatcttctc		

ors develop. Images were captured using soft ACT-2U by a Nikon camera.

DNA fragmentation analysis: DNA fragmentation analysis was performed according to a method described elsewhere (17). The cells harvested in cell invasion assays were centrifuged and washed twice in PBS to remove the supernatant. The pellets were suspended in 40 μ l of 10 mM EDTA, 50 mM Tris-HCl (pH 8.0) containing 0.5% NP40 and 20 U/ml proteinase K (Qiagen, Hilden, Germany), and incubated at 50°C for 1 h. RNase A (10 μ l of 1 mg/ml; Sigma-Aldrich) was added to each sample, and incubation at 50°C continued for 1 h. Samples were heated to 70°C for 5 min, and 10 μ l of 10 mM EDTA (pH 8.0) containing 1% low-melting-temperature agarose, 0.25% bromophenol blue, and 40% sucrose was mixed with each sample before loading into the dry wells of a 2% agarose gel containing 0.1 μ g/ml ethidium bromide. Electrophoresis was performed in 1 mM EDTA, 40 mM Tris-acetate (pH 7.8) at 80 V for 1.5 h. DNA bands were visualized and photographed under UV light.

Semi-quantitative RT-PCR: After infection with *S. typhimurium* BRD509 or B-pTIJEDIII, or mock infection, SK-N-SH cells were washed three times in PBS to remove extracellular bacteria, and treated with trypsin-EDTA buffer (0.25% trypsin, 0.5 mM EDTA). Total cellular RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Isolated RNA was quantified using spectrophotometry, aliquoted, and stored at -70°C until further use. First-strand cDNA was synthesized using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions; the primer for the synthesis of cDNA was Oligo(dT)₂₀ and 460 ng total RNA of each sample was used as a template. A 2- μ l volume of the synthesis products was used as a template in 10 μ l PCR reactions containing 10 \times PCR buffer, 1.5 mM MgCl₂, dNTPs (10 μ M/each), 1 U platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen), and 10 pM each of gene-specific forward and reverse primers for tumor necrosis factor receptor-associated death domain (TRADD) (18) and β -actin (Table 2). PCR parameters were optimized to obtain a single band of PCR products of the predicted size with the amount of amplified products in the linear range. The annealing temperature of all primers was 58°C, and 38 cycles of PCR were performed. The total PCR product was loaded on a 2% agarose gel with 10 \times loading buffer. After electrophoresis, the gel was stained using 10 μ g/mL ethidium bromide and analyzed using the Syngene image system.

RESULTS

Construction of recombinant strains: In SDS-PAGE and Western blot analysis, only protein bands of the predicted size (37 kDa) were detected in OM from *S. typhimurium* BRD509 or *E. coli* TOP10 (Fig. 2). This indicated that INPN-JEDIII can be produced in both strains. Also, intact B-pTIJEDIII stained positively (i.e., blue) using immunohistochemical staining, whereas BRD509 did not (data not shown). These

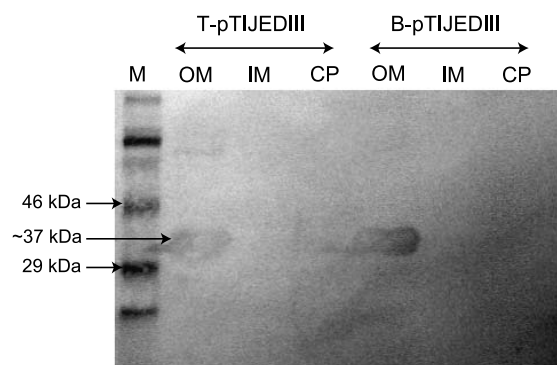


Fig. 2. Western blot analysis of cell fractions of T-pTIJEDIII and B-pTIJEDIII. M, Photomarkers™ Pre-stained Marker; OM, outer membrane fraction; IM, inner membrane fraction; CP, cytoplasmic fraction; T-pTIJEDIII, *E. coli* bearing pTIJEDIII; B-pTIJEDIII, *S. typhimurium* BRD509 bearing pTIJEDIII.

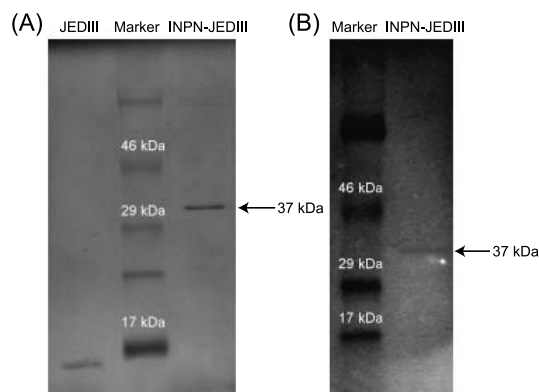


Fig. 3. (A) SDS-PAGE analysis of purified recombinant JEDIII and INPN-JEDIII proteins. (B) Western blot analysis of purified recombinant INPN-JEDIII protein.

results showed that recombinant strains to display JEDIII were successfully constructed.

Selection of target cells: Purified protein INPN-JEDIII and JEDIII were confirmed by Western blot and SDS-PAGE before cell-binding ELISAs were performed (Fig. 3). There was no evidence of a dose-titration effect of the three different concentrations of INPN-JEDIII and JEDIII protein used in cell-binding ELISAs. At each concentration, the level of binding of both proteins to BHK and Vero cells was higher than that to SK-N-SH cells ($P < 0.01$, Fig. 4 shows the binding activity at the high protein concentrations). There was no significant difference ($P > 0.05$) in the level of binding of INPN-JEDIII and JEDIII to SK-N-SH cells. Therefore, SK-N-SH cells were selected for further study to avoid the potential influence of the binding activity of the displayed protein on the invasiveness of the recombinant bacterium.

Whole-cell ELISA measurement of the amount of intracellular *Salmonella* antigen released after the infection of SK-N-SH cells with B-pTIJEDIII or BRD509 confirmed that the

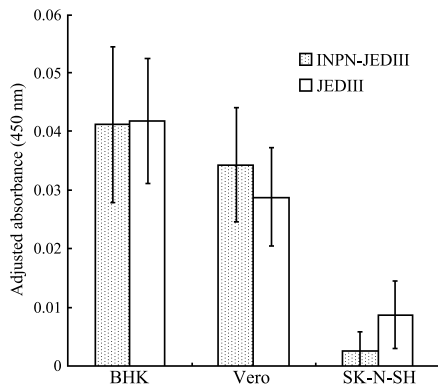


Fig. 4. BHK, Vero, and SK-N-SH cells binding of purified recombinant INPN-JEDIII and JEDIII proteins at high concentrations (440 ng and 200 ng, respectively) measured by cell-binding ELISA.

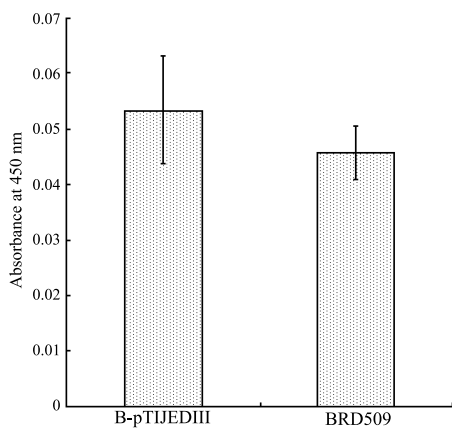


Fig. 5. Release of intracellular *Salmonella* antigen from SK-N-SH cells infected with BRD509 or B-pTIJEDIII measured using whole-cell ELISA.

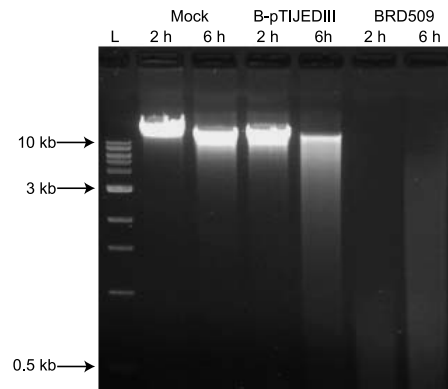


Fig. 6. DNA fragmentation analysis of total cellular DNA extracted from SK-N-SH cells after mock infection or infection with B-pTIJEDIII or BRD509.

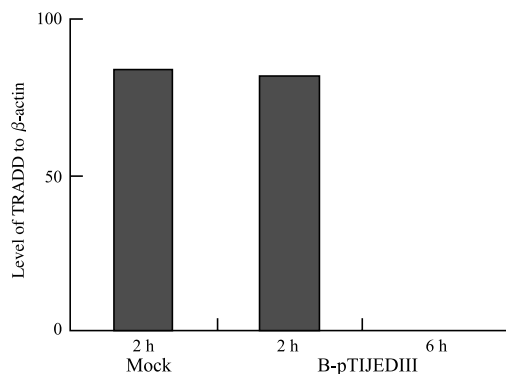


Fig. 7. Semi-quantitative RT-PCR analysis of TRADD mRNA expression in SK-N-SH cells at 2 h post-infection with B-pTIJEDIII compared with mock infection.

display of JEDIII did not increase the invasiveness of the recombinant bacterium (Fig. 5).

Effects of infection with recombinant bacterium on SK-N-SH cells: After infection with B-pTIJEDIII, the SK-N-SH cells retained their spindle-like shape, whereas after infection with BRD509, the cells rounded up, suggesting severe cell damage. Total DNA extracted from SK-N-SH cells infected with B-pTIJEDIII exhibited a smear on agarose gels, which is suggestive of necrosis rather than apoptosis (19). The amount of smearing was higher at 6 h post-infection than that at 2 h post-infection. The mock-infected cells had an almost-intact DNA band at 2 h post-infection and a slight smear at 6 h post-infection. Cells infected by *S. typhimurium* BRD509 exhibited apparent smears on gels at 2 h or 6 h post-infection. Significantly, the smear level of cells infected by BRD509 at 2 h post-infection was the highest compared with all other samples (Fig. 6). The results indicate that either B-pTIJEDIII or BRD509 can cause SK-N-SH cells to become necrotic, with the latter bacterial strain having a more adverse effect.

Semi-quantitative two-step RT-PCR was used to investigate the influence of infection on the gene of apoptosis-associated TRADD. The relative expression of TRADD to the β -actin housekeeping gene is shown in Fig. 7. This shows the results of one assay, which were confirmed by a second independent assay. Notably, the level of TRADD was equivalent at 2 h in mock- and B-pTIJEDIII-infected cells. No TRADD was detected at 6 h post-infection in B-pTIJEDIII-infected cells.

DISCUSSION

Virus infection can have opposing effects on the apoptosis of host cells, either blocking it or increasing it. The latter often occurs at the late stage of infection, whereas the former occurs at the early stage, during the absorption and penetration of the virus. The E protein of JEV is involved in receptor binding and membrane fusion; consequently, it may exert some inhibitory effects on apoptosis. It was reported that the JEV YL strain or its entire E protein induce apoptosis in target cells, whereas domain III alone did not have this effect. The apoptosis of cells occurred at least 24 h after infection or transfection with the entire E protein gene (20). Even in SK-N-SH cells, a cell line sensitive to apoptosis induction, apoptosis occurred at least 6 h after JEV infection (21), when virus entry had been completed. In this study, we demonstrated the potential utility of using recombinant *S. typhimurium* expressing domain III of JEV to indirectly study apoptosis induced by JEV.

Salmonella strains can rapidly kill host cells (22), and the mechanism of cell damage is very special—neither typical apoptosis nor typical necrosis (23–26). Though manifesting some necrotic characters, it is both programmed-cell-death- and caspase-dependent (22,23,27,28). We used *S. typhimurium* BRD509 to rapidly induce necrosis in SK-N-SH cells, and then examined the effects of expressing a JEV protein on necrosis to simulate its effects on the apoptosis of host cells during virus absorption and penetration. The use of the attenuated vaccine strain of *S. typhimurium*, BRD509, meant

that these studies of JEV pathogenesis did not require biosafety level 3 facilities. Because the entire E protein can induce apoptosis (20), domain III alone was selected as the display protein. Thus, cell damage was completely caused by the bacterial strain.

It has been reported that the E protein of JEV has glycosaminoglycan (GAG)-binding motifs, mainly located in domain III (16). To avoid GAG-binding activity of the displayed protein that would influence the invasiveness of *Salmonella*, we selected a cell line with a low affinity for GAG (SK-N-SH) using a cell-binding ELISA. Whole-cell ELISA, rather than counting colony-forming units (CFUs), was used to assess the bacterial invasiveness of SK-N-SH cells. Gentamicin may inhibit or kill intracellular bacteria (29), and small colony variants (SCV) are released after gentamicin treatment (30); therefore, using the CFU count to assess invasion frequency may be inaccurate. The equivalent release of *Salmonella* antigen after infection with BRD509 or B-pTIJEDIII confirmed that the display of domain III of JEV E protein does not significantly influence bacterial invasiveness. This suggested that infection was performed under conditions of equal invasion efficiency for the recombinant and parental strains, and any differences in the level of necrosis may be ascribed to delivering domain III of JEV E protein. Because it was shown in the cell-binding ELISA that INPN seemed not to improve the binding activity of JEDIII, we postulated that anti-necrotic effects were mainly due to JEDIII itself. However, our study did not generate direct evidence to exclude the interference of INPN. Although our approach allows us to study the effects of one protein independently of other JEV proteins, this in itself leads to some shortcomings, as with all model systems. For example, the system does not allow the cellular responses to the biosynthesis of E proteins to be examined; therefore, the similarity between the effects on apoptosis observed in this system and those observed when JEV infects host cells is likely to be limited to the early stages of absorption and penetration.

It has been reported that apoptosis induced by JEV is regulated by the expression of TRADD. The expression of TRADD can increase viral load and glial cell inflammation (21,31). Prior to typical apoptosis, the expression of TRADD increases first; its transcript levels increased significantly 6 h and 9 h after JEV infection (21). In contrast, in our study, transcriptional levels of TRADD seemed to decrease 6 h after infection by *S. typhimurium* displaying domain III of JEV E protein. Whether this is a result of the putative anti-apoptotic effect of domain III of JEV E protein on virus infection directly influencing the expression of TRADD should be investigated further, but this possibility is supported by other published data (21).

In conclusion, it is feasible to study the pathogenesis of JEV using a recombinant bacterium displaying viral protein.

ACKNOWLEDGMENTS

We thank Dr. Lin Li for providing some materials and technical suggestions. Our thanks also go to Dr. Derek Pickard for providing *Salmonella typhimurium* BRD509.

This project was supported by a grant from the Chinese Academy of Sciences (KSCX1-YW-R-07).

REFERENCES

1. Solomon, T. (2006): Control of Japanese encephalitis—within our grasp? *N. Engl. J. Med.*, 355, 869-871.
2. Mackenzie, J.S., Gubler, D.J. and Petersen, L.R. (2004): Emerging

- flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. *Nat. Med.*, 10, S98-109.
3. Knipe, D.M.H. and Peter, M. (2006): *Fields Virology*. 5th ed. vol. 1. Lippincott Williams & Wilkins, Pa., USA.
4. Heinz, F.X. (1986): Epitope mapping of flavivirus glycoproteins. *Adv. Virus Res.*, 31, 103-168.
5. Wu, K.P., Wu, C.W., Tsao, Y.P., et al. (2003): Structural basis of a flavivirus recognized by its neutralizing antibody: solution structure of the domain III of the Japanese encephalitis virus envelope protein. *J. Biol. Chem.*, 278, 46007-46013.
6. Rey, F.A. (2003): Dengue virus envelope glycoprotein structure: new insight into its interactions during viral entry. *Proc. Natl. Acad. Sci. USA*, 100, 6899-6901.
7. Rey, F.A., Heinz, F.X., Mandl, C., et al. (1995): The envelope glycoprotein from tick-borne encephalitis virus at 2 Å resolution. *Nature*, 375, 291-298.
8. Luria-Perez, R., Cedillo-Barron, L., Santos-Argumedo, L., et al. (2007): A fusogenic peptide expressed on the surface of *Salmonella enterica* elicits CTL responses to a dengue virus epitope. *Vaccine*, 25, 5071-5085.
9. Fu, G., Wijburg, O.L., Cameron, P.U., et al. (2005): *Salmonella enterica* serovar Typhimurium infection of dendritic cells leads to functionally increased expression of the macrophage-derived chemokine. *Infect. Immun.*, 73, 1714-1722.
10. Dunstan, S.J., Simmons, C.P. and Strugnell, R.A. (2003): In vitro and in vivo stability of recombinant plasmids in a vaccine strain of *Salmonella enterica* var. Typhimurium. *FEMS Immunol. Med. Microbiol.*, 37, 111-119.
11. Jung, H.C., Lebeault, J.M. and Pan, J.G. (1998): Surface display of *Zymomonas mobilis* levansucrase by using the ice-nucleation protein of *Pseudomonas syringae*. *Nat. Biotechnol.*, 16, 576-580.
12. Kwak, Y.D., Yoo, S.K. and Kim, E.J. (1999): Cell surface display of human immunodeficiency virus type 1 gp120 on *Escherichia coli* by using ice nucleation protein. *Clin. Diagn. Lab. Immunol.*, 6, 499-503.
13. Wu, M.L., Tsai, C.Y. and Chen, T.H. (2006): Cell surface display of Chi92 on *Escherichia coli* using ice nucleation protein for improved catalytic and antifungal activity. *FEMS Microbiol. Lett.*, 256, 119-125.
14. Li, L., Kang, D.G. and Cha, H.J. (2004): Functional display of foreign protein on surface of *Escherichia coli* using N-terminal domain of ice nucleation protein. *Biotechnol. Bioeng.*, 85, 214-221.
15. Shi, H. and Wen Su, W. (2001): Display of green fluorescent protein on *Escherichia coli* cell surface. *Enzyme Microb. Technol.*, 28, 25-34.
16. Wu, S.C., Chiang, J.R. and Lin, C.W. (2004): Novel cell adhesive glycosaminoglycan-binding proteins of Japanese encephalitis virus. *Biomacromolecules*, 5, 2160-2164.
17. Uehara, T., Miyawaki, T., Ohta, K., et al. (1992): Apoptotic cell death of primed CD45RO+ T lymphocytes in Epstein-Barr virus-induced infectious mononucleosis. *Blood*, 80, 452-458.
18. George, J., Gondi, C.S., Dinh, D.H., et al. (2007): Restoration of tissue factor pathway inhibitor-2 in a human glioblastoma cell line triggers caspase-mediated pathway and apoptosis. *Clin. Cancer Res.*, 13, 3507-3517.
19. Abdel-Latif, L., Murray, B.K., Renberg, R.L., et al. (2006): Cell death in bovine parvovirus-infected embryonic bovine tracheal cells is mediated by necrosis rather than apoptosis. *J. Gen. Virol.*, 87, 2539-2548.
20. Chen, S.O., Chang, T.J., Stone, G., et al. (2006): Programmed cell death induced by Japanese encephalitis virus YL vaccine strain or its recombinant envelope protein in varied cultured cells. *Intervirology*, 49, 346-351.
21. Swarup, V., Das, S., Ghosh, S., et al. (2007): Tumor necrosis factor receptor-1-induced neuronal death by TRADD contributes to the pathogenesis of Japanese encephalitis. *J. Neurochem.*, 103, 771-783.
22. van der Velden, A.W., Velasquez, M. and Starnbach, M.N. (2003): *Salmonella* rapidly kill dendritic cells via a caspase-1-dependent mechanism. *J. Immunol.*, 171, 6742-6749.
23. Boise, L.H. and Collins, C.M. (2001): *Salmonella*-induced cell death: apoptosis, necrosis or programmed cell death? *Trends Microbiol.*, 9, 64-67.
24. Brennan, M.A. and Cookson, B.T. (2000): *Salmonella* induces macrophage death by caspase-1-dependent necrosis. *Mol. Microbiol.*, 38, 31-40.
25. Knodler, L.A. and Finlay, B.B. (2001): *Salmonella* and apoptosis: to live or let die? *Microbes Infect.*, 3, 1321-1326.
26. Monack, D.M., Raupach, B., Hromockyj, A.E., et al. (1996): *Salmonella typhimurium* invasion induces apoptosis in infected macrophages. *Proc. Natl. Acad. Sci. USA*, 93, 9833-9838.
27. Grant, A.J., Sheppard, M., Deardon, R., et al. (2008): Caspase-3-

- dependent phagocyte death during systemic *Salmonella enterica* serovar Typhimurium infection of mice. *Immunology*, 125, 28-37.
28. Schauser, K., Olsen, J.E. and Larsson, L.I. (2005): *Salmonella* Typhimurium infection in the porcine intestine: evidence for caspase-3-dependent and -independent programmed cell death. *Histochem. Cell Biol.*, 123, 43-50.
 29. Drevets, D.A., Canono, B.P., Leenen, P.J., et al. (1994): Gentamicin kills intracellular *Listeria monocytogenes*. *Infect. Immun.*, 62, 2222-2228.
 30. Cano, D.A., Pucciarelli, M.G., Martinez-Moya, M., et al. (2003): Selection of small-colony variants of *Salmonella enterica* serovar Typhimurium in nonphagocytic eucaryotic cells. *Infect. Immun.*, 71, 3690-3698.
 31. Swarup, V., Ghosh, J., Das, S., et al. (2008): Tumor necrosis factor receptor-associated death domain mediated neuronal death contributes to the glial activation and subsequent neuroinflammation in Japanese encephalitis. *Neurochem. Int.*, 52, 1310-1321.