A MODIFIED IMMUNOFLUORESCENCE ASSAY FOR DETECTION OF JAPANESE ENCEPHALITIS VIRUS-INFECTED CELLS

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Summary. – Japanese encephalitis virus (JEV) infections are currently detected by indirect immunofluorescence (IF) assay using virus-specific antibodies on acetone-fixed smears. On few occasions, the acetone treatment was reported to damage certain epitopes on JE virus (JEV) glycoprotein. Here, we have made an attempt to adopt quick paraformaldehyde fixation followed by a short detergent treatment of cells in suspension for identification of JEV-infected brain cells of laboratory-reared *Toxorhynchitis splendens* mosquito larvae using virus-specific antibodies. JEV-positive cells could be scored by the presence of a well defined intracellular immunofluorescence staining against unstained uninfected antibody-treated cells. The advantage of this assay is that stained cell suspensions can be stored for up to 4 weeks, allowing analysis at convenience. Thus, the modified IF assay can be employed as an additional/alternate technique to standard IF assay for detection of JEV in cells and also to screen hybridoma cell lines for anti-JEV antibody production.

Key words: antibodies; immunofluorescence; assay; Japanese encephalitis virus; mosquito larval brain cells; paraformaldehyde; saponin

In India, JE is one of the public health problems in three southern states, namely Tamil Nadu, Karnataka and Andhra Pradesh (Reuben and Gajanana, 1997). JEV has bee found responsible for most of viral encephalitides in some of the districts of Tamil Nadu (Reuben and Gajanana, 1997; Kabilan *et al.*, 2000). A number of laboratory tests are available for the diagnosis of JE (Gajanana *et al.*, 1996; Maha and Igarashi, 1997). In general, JEV antigen is detected in host cells by IF assay using acetone-fixed cell smears, virus-specific antibodies (polyclonal or monoclonal) and antibodies conjugated with fluorescein isothiocyanate (FITC, Mourya, 1990). Although IF assay is a reliable method for the diagnosis of JE, there have been difficulties with monoclonal antibodies (MAbs). This might be due to the acetone treatment which has been reported to denature a few specific epitopes on JEV E glycoprotein (Gupta *et al.*, 1993, 2000).

In this study, suspensions of brain cells from mosquito *Tx. splendens* larvae inoculated with JEV were fixed with 4% paraformaldehyde followed by a rapid saponin treatment to permeabilize the cell membrane for the specific antibodies to recognize and interact with the specific cytokine, while retaining its antigenicity (Henter *et al.*, 1988).

Cells and smears were prepared as follows. Sixteen late third instar *Tx. splendens* larvae from a laboratory cycling colony were inoculated intracerebrally (Mourya, 1990) with about 10 PFU of JEV in 17 µl of mouse brain suspension diluted in 0.75% bovine serum albumin in phosphate-buffered saline pH 7.4 (BAPS). Control larvae were inoculated with BAPS. The larvae were reared for 8 days at 29°C. Eight larvae were used for each IF assay. From each larva, two head squeeze smears were fixed with cold acetone for 5 mins and used for standard IF assay. In parallel, brain

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Abbreviations: BAPS = 0.75% bovine serum albumin in PBS; IF = immunofluorescence; JE = Japanese encephalitis; JEV = JE virus; FITC = fluorescein isothiocyanate; MAb = monoclonal antibody; PBS = phosphate-buffered saline pH 7.4; UV = ultraviolet

cells from 8 larvae were pooled in phosphate-buffered saline pH 7.4 (PBS), partitioned in two halves and used in the modified IF assay. Cells from control uninfected larvae were processed similarly.

The standard IF assay was performed as follows. Acetone-fixed smears were dried and incubated with a drop of rabbit anti-JEV immune serum (1:100) and a drop of 1:100 dilution of FITC-conjugated swine anti-rabbit immunoglobulins (Dakopat, Denmark), and viewed under ultra violet (UV) microscope. All incubations were done in a humidified chamber at room temperature for 30 mins followed by washing thrice in PBS. If any of the smears was found to be positive with the rabbit anti-JEV immune serum, the duplicate smears would be incubated with JEV-specific MAb 112 (kind gift from Dr. J.K. Kiroda, Tokyo Metropolitan Institute of Neurosciences, Tokyo, Japan) and FITC-conjugated rabbit anti-mouse immunoglobulins (Dakopat, Denmark), and viewed under UV microscope (Gajanana *et al.*, 1996).

The modified IF assay was carried out in tubes (Kabilan *et al.*, 1990) as follows. The washed cells in suspension were fixed with 4% paraformaldehyde solution (paraformaldehyde 40 g/l, NaH_2PO_4 x H_2O 16.833 g/l, NaOH 3.85 g/l, and glucose 5.4 g/l, pH 7.4) for 5 mins at room temperature After washing twice with PBS, the cells were resuspended in 50 µl of PBS and incubated for 30 mins at room temperature with 50 µl of rabbit anti-JEV immune serum (1:100 dilution in PBS) supplemented with 0.1% saponin (Sigma). Subsequent incubations and washings were performed with PBS containing 0.1% saponin. The cells were then incubated with a 1:100 dilution of FITC-labeled swine anti-rabbit immunoglobulins for 30 mins at room temperature, washed twice, resuspended in 30 µl of PBS and a drop of the cell suspension was subjected to UV microscope examination. If the sample was found to be positive, the same procedure was repeated with JEV-specific MAb.

JEV infected cells were visualized by the presence of intracellular fluorescence staining, while the uninfected cells in the same smear gave no fluorescence. No fluorescent staining was observed in cells from control, uninfected larvae. The infected cells showed a smooth intracellular fluorescence. The nuclei appeared to be stained in some cells in a distinct manner as compared to the cytoplasmic staining. The fluorescence staining was intense and well defined with minimal background. The virus-infected cells were easily identified with certainty. A weak staining/JEV positivity could be scored in larval brain cells inoculated with 10 times less virus dose (about 1 PFU).

While fixatives such as acetone and ethanol preserve sometimes the antigenicity and not the cellular morphology (Sandvir *et al.*, 1987), aldehyde fixation preserves often both the cellular morphology and antigenicity (Henter *et al.*, 1988). However, the aldehyde fixation involves an additional step of detergent treatment to permeabilize the cell membrane. IF assay on acetone-fixed cell smears often successfully detects JEV infections. On few occasions, acetone fixation has led to the loss of epitopes of some viral antigens present in the cytoplasm (Buckley and Gould, 1988) including E glycoproteins of JEV (group II strains) (Gupta *et al.*, 1993, 2000).

Thus, our experience with this assay is that the sequential exposure of the cells to the fixative and detergent has preserved the antigenicity and led to minimal cell aggregation.

Although we are not claiming any superiority of the modified assay over the standard assay using different fixatives, there are a few advantages of the former assay: first, the processing of brain cells collected from a number of *Tx. splendens* larvae inoculated with the same test sample will save time, manpower and biological reagents; second, the stained cell preparations can be stored without observable changes at least for 4 weeks in the refrigerator for later analysis.

The recognition of different antigenicities of various strains of JEV remains to be compared using the modified and standard IF assay with a number of JEV-specific MAbs to elucidate the effect of paraformaldehyde fixation on the epitopes. However, using the modified assay, we could detect JEV-positive cells in cerebrospinal fluid (data not shown) collected from children with acute encephalitis syndrome (Kabilan *et al.*, 2000). Sharp staining of the virus observed in the modified assay does not require experienced UV microscopists to score the positivity.

Thus, we believe that this simple, specific and rapid assay can be adopted as an additional/alternate method to standard IF assay to detect JEV-infected cells of human or mosquito origin using JEV-specific monoclonal/polyclonal antibodies.

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