POTENCY TESTING OF INACTIVATED TISSUE CULTURE VACCINE AGAINST JAPANESE ENCEPHALITIS USING ANTIGEN CAPTURE ELISA

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Summary. – The potency of vaccines against Japanese encephalitis (JE) is usually determined by a mouse challenge test. In the present study, an antigen capture enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies (MoAbs) was used to screen inactivated tissue culture JE vaccine lots. This test is simple, quick and reliable besides being very sensitive and specific.

Key words: Japanese encephalitis virus; tissue culture; inactivated vaccine; antigen capture ELISA; screening test

JE has emerged as a major health problem on the Asian continent. As many as 35,000 cases and 10,000 deaths are reported annually (Tsai and Yu, 1994). In India, JE outbreaks have occurred in different parts of the country and case fatality rates of 20 – 50% have been recorded (Rodrigues, 1984). Among different strategies for the control of this disease, an immunization has proved to be a practical and useful approach (Igarashi, 1993).

In our laboratoty, tissue culture-derived inactivated JE vaccines were developed by employing the microcarrier technology in a 2-liter-laboratory fermentor (B. Braun) as well as the classical monolayer technique in Roux bottles. The potency testing of these vaccines has been usually done by a mouse challenge method (Savant, 1987). Its disadvantage is the fact that it requires a large number of mice and its results could be obtained only after 21 days.

An antigen capture ELISA has been used for the identification of dengue virus (Kuno *et al.*, 1985). In the present study, this kind of ELISA was developed as a screening test for the determination of potency of JE vaccine lots so as to reduce the number of mice required for the test as well as the time required for testing.

Vaccine lots were prepared in two systems, namely in Roux bottles and in laboratory scale fermentor using the microcarrier technology (van Wezel, 1967).

Briefly, mycoplasma free, non-tumourigenic Vero cells were cultured in Roux bottles using Eagle's Minimal Essential Medium (MEM) with Earle's salts and 10% goat serum. Confluent monolayers were infected with JE virus (JEV, Nakayama strain) at a multiplicity of infection (MOI) of 0.01. Soon after the cytopathic effect (CPE) appeared, the tissue culture fluid was harvested, clarified by centrifugation and inactivated with formalin diluted 1:2000 at 4°C for 30 days. Some vaccine lots were prepared by adding 0.05 mol/l Mg^{++} ions during adsorption of the virus onto cells and by incorporating 0.02% gelatin as a stabiliser in the virus growth medium.

The vaccine was made in the fermentor as follows. Vero cells were grown on Cytodex (Pharmacia) microcarriers in 1000 ml of MEM with 10% goat serum. Confluent monolayers were infected with JEV at MOI of 0.01. After CPE appeared, the tissue culture fluid was harvested, clarified and inactivated as mentioned above. Some lots were prepared by adding 0.05mmol/l Mg^{++} ions during adsorption of the virus onto cells and by incorporating 0.02% gelatin in the virus growth medium. Vaccine lots were made using 1 g/l and 3 g/l Cytodex.

After inactivation for 30 days, vaccine lots were tested for the sterility and presence of residual infectivity in infant mice. For the

Abbreviations: CPE = cytopathic effect; ELISA = enzyme-linked immunosorbent assay; HI = haemagglutination-inhibition; i.c. = intracerebral(ly); i.p. = intraperitoneal(ly); JE = Japanese encephalitis; JEV = JE virus; MEM = Eagle's Minimal Essential Medium; MoAb = monoclonal antibody; MOI = multiplicity of infection; PBS = phosphate-buffered saline; PrI = protective index

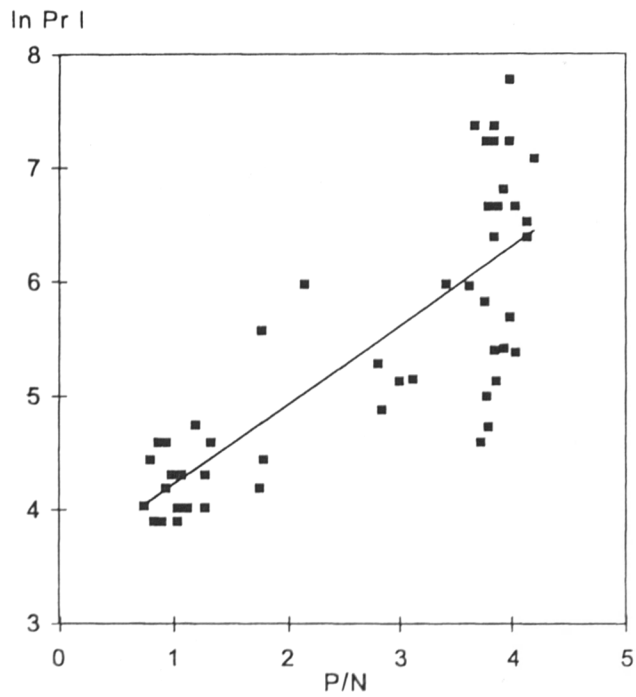

Fig. 1
Correlation between PrI and P/N of the antigen capture ELISA

Table 1. P/N ratios of JE vaccine lots captured by MoAbs Hs-1 and Hx-2

	P/N	
Vaccine lot	MoAb Hs-1	MoAb Hx-2
93-23	3.215	2.968
93-28	2.705	1.764
93-31	3.345	6.708
Reference vaccine	0.671	6.101

potency they were tested in mice as follows. Briefly, groups of 10 mice were immunized with serial dilutions of the vaccine made in phosphate-buffered saline (PBS) pH 7.2. Two 0.5 ml doses were administered to mice on days 0 and 3 by the intraperitoneal (i.p.) route. The mice were challenged i.p. with 100 – 1000 LD_{50} of JEV in 0.2 ml. The JEV stock was prepared by grinding brains harvested from JEV-infected infant mice. After 3 hrs, 0.02 ml of 1% starch was injected intracerebrally (i.c.) into each mouse to break the blood-brain barrier (Savant, 1987). A control group, included in each test, consisted of non-immunized mice. The mice were observed for 14 days for sickness and mortality. The protective index (PrI), which is the reciprocal of that dilution which shows 50% protection for a given challenge dose, was calculated by the Spearman-Karber's method. The mouse brain vaccine produced in Kasauli, India, was used as reference vaccine.

The antigen capture ELISA was carried out utilising MoAbs against JEV, which were developed at National Institute of Virology, Pune, against a JEV strain isolated from West Bengal in 1973 (Bankura strain NIV No. 733913).

Two types of MoAbs were employed, Hs-1 and Hx-2. MoAb Hs-1 of the IgG type, showed strong haemagglutination-inhibition (HI) and neutralizing activity and was JEV-specific in HI test. MoAb Hx-2 also showed HI and neutralizing activity but was cross-reactive with other flaviviruses such as West Nile and dengue viruses in HI test (Kedarnath *et al.*, 1986; Cecilia *et al.*, 1988; Ghosh *et al.*, 1989). The MoAbs diluted in carbonate buffer were added in 1 µg per well and incubated overnight at 4°C in wells. On the following day, the MoAbs were discarded and the wells were filled with 1% bovine serum albumin (BSA) in PBS to block unoccupied sites. Then the wells were washed thrice with a wash buffer and various tissue culture vaccine lots were added. A Vero cell culture supernatant was used as a negative control. The purified JEV antigen and the reference mouse brain vaccine were included as positive controls. The wells were incubated overnight at 4°C, washed thoroughly and suitably diluted Hs-1 and Hx-2 biotinylated conjugates (50 µl) were added. The wells were incubated at 37°C for 1.5 hrs. After removing the conjugates and thorough washing, 50 µl of an avidin-horseradish peroxidase conjugate was added to each well. The reaction proceeded at 37°C for 30 mins. Then 200 µl of the substrate prepared in citrate-phosphate buffer was added. The wells were left in the dark for about 4-5 mins after which the reaction was stopped by adding 100 µl of 4 N sulfuric acid and the absorbance at 492 nm (A_{492}) was read in a Titertek ELISA reader. The ratio P/N of the A_{492} of the vaccine (P) to that of the negative control (N) was calculated and considered positive if equal or higher than 2.00.

The tested tissue culture vaccine lots reacted similarly with both MoAbs Hs-1 and Hx-2, while the reaction of the reference vaccine was much stronger with MoAb Hs-1. The results of a few vaccine lots are given in Table 1.

It appears from the data summarized in Table 1 that tissue culture vaccine lots No. 93-23, 93-28 and 93-31 reacted both with the specific MoAb (Hs-1) and the cross-reactive MoAb (Hx-2). High A_{492} readings were recorded for all three vaccine lots captured by MoAb Hs-1 giving corresponding high P/N ratios. In contrast, the A_{492} readings for MoAb Hx-2 were lower. However, the P/N ratios were positive in both cases. The reference vaccine which served as a positive control showed a low reactivity with MoAb Hs-1 and a negative P/N ratio but reacted strongly with MoAb Hx-2 giving a high P/N ratio.

Since the tissue culture vaccine reacted better with MoAb Hs-1 than with MoAb Hx-2, the former MoAb was used as reagent to screen different vaccine lots. The natural logarithms of PrI of the vaccine lots were plotted on the

ordinate against the corresponding P/N ratios on the abscissa in Fig. 1. A linear relationship was observed between them (ln y = 3.530684 + 0.708308 x). This relationship was found highly significant by the t-test.

During the vaccine production, when a large number of vaccine lots are to be tested, it would be possible to distinguish the high potency lots from the poor potency ones using this test. In other words, this test can be used as a rapid screening test for the vaccine potency thereby reducing the number of experimental animal required for the testing. Only the high potency lots according to the antigen capture ELISA would be subsequently tested in mice.

ELISA has gained a popularity in the diagnosis of viral diseases because of its simple, sensitive and specific nature. In the present study, the antigen capture ELISA was employed for evaluating the potency of JE vaccine lots. The antigen in the vaccine lots was captured by a MoAb, making the test extremely specific. The biotinylated conjugates contributed to the sensitivity of the assay. The results were obtained far more rapidly than in virus titrations or potency tests.

It was observed that the tissue culture vaccine reacted both with JEV-specific (Hs-1) and cross-reactive (Hx-2) MoAbs. The reference vaccine reacted only with the the cross-reactive MoAb (Hx-2). This indicates that both the Hs-1 and Hx-2 epitopes were present on the tissue culture vaccine JEV Nakayama strain, while only the Hx-2 epitope was present on the reference vaccine JEV strain. These results are consistent with those of Ghosh *et al.* (1989). These authors further suggested that since the Hs-1 epitope is associated with the HI and neutralization (*in vitro* and *in vivo*) but not with the antibody-dependent plaque enhancement, its presence in a vaccine strain should be a decisive factor while choosing different strains for vaccine production. An earlier work by Takegami *et al.* (1982) also suggested that strain-specific antigenic sites of JEV Nakayama strain E protein are more effective in inducing the production of neutralizing antibodies than the common antigenic sites. Brandt (1990) also gives similar recommendations.

Summing up, we were able to correlate PrI and P/N ratios of the antigen capture ELISA of various tissue culture JE vaccine lots. Thus, the antigen capture ELISA may be used as a preliminary screening test for the selection of those vaccine lots which are worth of subsequent evaluation in a protective *in vivo* test.

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