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ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF SERUM ANTIBODY IN CHILDREN VACCINATED WITH JAPANESE ENCEPHALITIS VACCINE

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SUMMARY Serum antibodies in children who had been vaccinated with Japanese encephalitis (JE) vaccine were measured by enzyme-linked immunosorbent assay (ELISA) and neutralization (N) and hemagglutination-inhibition (HI) tests. Of 20 serum samples obtained after two shots of JE vaccine in the first year, all but one showed positive titers in the ELISA and N test, but five showed negative titers in the HI test. All 12 serum samples obtained after booster immunization with JE vaccine in the second year showed positive and considerably higher titers in all three tests. Moreover, a high correlation was found between the ELISA, N and HI titers. These results indicate that the ELISA is useful for detecting antibodies in subjects immunized with JE vaccine.

INTRODUCTION

We have studied on the effectiveness of Japanese encephalitis (JE) vaccine for inducing an antibody response to JE virus (Quina et al., 1978; Susilowati et al., 1981; Juang et al., 1983; Yamada et al., 1986). In these studies, we mainly used the neutralization test because it is the most specific and sensitive serological test for detection of serum antibodies against JE virus. However, the test requires special facilities and equipment, and is time consuming. Recently, the ELISA developed by Engvall and Perlman (1971) has been widely used for serodiagnosis of viral infections. This ELISA is sensitive and can be used to examine many specimens at the same time. Since there have been numerous outbreaks of JE in many Asian countries, and especially South-east Asian countries, China and India (Umenai et al., 1985), plans are afoot to use JE vaccine in several countries and it will be important to measure the antibody levels of the vaccinees. In the present study, we compared the ELISA with the neutralization and hemagglutination-inhibition (HI) tests using serum samples from children who had been vaccinated with the JE vaccine.

MATERIALS AND METHODS

1. *Vaccination and blood sampling*

Purified inactivated JE vaccine (Takaku et al., 1968) was obtained from the Research Foundation for Microbial Diseases of Osaka University. The vaccine was administered to children who visited the Vaccine Clinic of the Department of Pediatrics, School of Medicine, Osaka University, in volumes of 0.5 ml for 1–3 year olds and 1.0 ml for older children. In the present study, serum samples were collected from three groups of children who had no history of previous JE vaccination and whose sera contained no detectable neutralization antibody before vaccination. The first group consisted of 32 children (2–5 year olds) from whom blood was taken before JE vaccination (Serum I). The second group consisted of 20 children (2–5 year olds) from whom blood was taken 1–3 months after two shots of JE vaccine in the first year (Serum II). The third group consisted of 12 children (1–8 year olds) who had received two shots of JE vaccine in the previous year, and from whom blood was taken 1–3 months after one booster dose of JE vaccine in the second year (Serum III).

2. *ELISA*

ELISA was performed by the method of Igarashi et al. (1981) with slight modifications. The wells of 96-well flat-bottom microplates (Linbro; Flow Laboratories, Inc., McLean, Va., U.S.A.) were coated with 100 μl per well of JE vaccine at a dilution of 1: 50 in 0.1 *M* carbonate-bicarbonate buffer (pH 9.5) which was used as virus antigen in the ELISA. After overnight incubation at 4 C, the plates were washed five times with PBS-T (0.1% Tween 20 in phosphate buffered saline, pH 7.5) for several seconds each time. Then, 50 μl of the blocking solution (PBS-0.5% bovine serum albumin) was introduced and the wells were incubated for 30 min at room temperature. Thereafter, the plates were treated successively with the following solutions: (i) Test serum (50 μl per well) serially diluted two-fold in PBS-T at 37 C for 1 h. (ii) β-galactosidase conjugated anti-human IgG goat serum (50 μl per well, Zymed Laboratories, Burlingame, Ca., U.S.A.) diluted 1: 500 in PBS-T containing 10 m*M* $MgCl_2$ and 0.1 *M* 2-mercaptoethanol at 37 C for 1 h. (iii) Substrate; 2-N phenyl-β-D galactopyranoside (100 μl per well, Wako Pure Chemicals Co. Osaka) diluted to 1 mg/ml in PBS containing 10 m*M* $MgCl_2$ and 0.1 *M* 2-mercaptoethanol at 37 C for 1 h. (iv) Stopping solution (1 *M* Na_2CO_3, 50 μl per well) at room temperature for 15 min. After steps (i) and (ii), the plates were washed as described above. The optical density (OD) at 405 nm was measured with a Titertek Multiskan (Dynateck Laboratories, Inc., Alexandria, Va. U.S.A.). The ELISA titer of each test serum was calculated from the curve drawn by plotting the OD values of the serially diluted serum. At high dilutions, the ODs showed a constant low value, which represented a non-specific reaction, and the ELISA titer was expressed as the reciprocal of the serum dilution that gave an OD of 3 times the constant value.

3. *Neutralization (N) test*

The neutralizing antibody titers of the serum specimens were determined by an improved rapid focus reduction neutralization test in 96-well tissue culture plates by the PAP (peroxidase-antiperoxidase) staining technique (Okuno et al., 1985). The Nakayama-Yoken strain, the same strain as for the JE vaccine, was used in the test. Titers were expressed as 50% focus reduction units, the reciprocal of the serum dilution that reduced the number of foci to 50% of the control value. Serum specimens were heat-inactivated at 56 C for 30 min before the test.

4. *Hemagglutination-inhibition (HI) test*

The hemagglutination-inhibition (HI) test was performed by the method of Clark and Casals (1958) as modified for microtiter systems (Sever, 1962).

RESULTS

1. *Distribution of ELISA titers in Serum I*

Thirty-two serum samples (Serum I) that had no detectable antibodies by the neutralization and HI tests were examined by the ELISA (Fig. 1). One serum sample showed an ELISA titer of 160, but the titers of all the others were less than 100, and the peak titer was 20. From these results, we designated ELISA titers of less than 100 as negative, and those of more than 100 as positive.

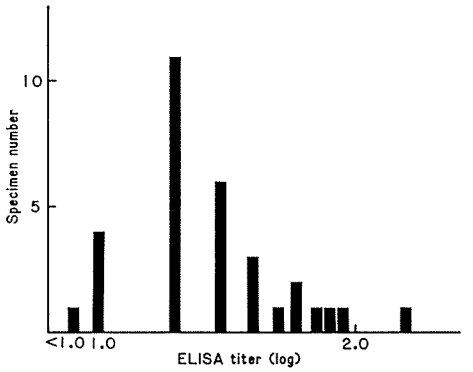


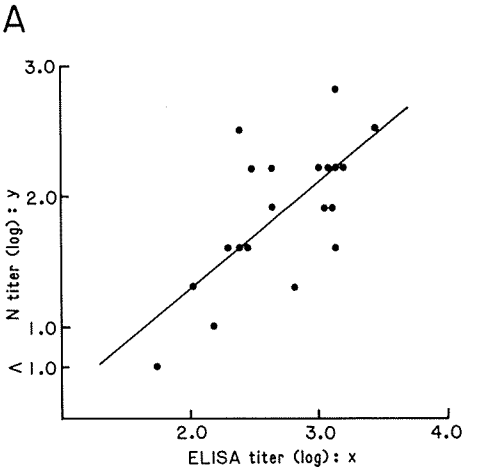
FIGURE 1. Distribution of ELISA titers in serum samples that had negative antibody titers by the N test.

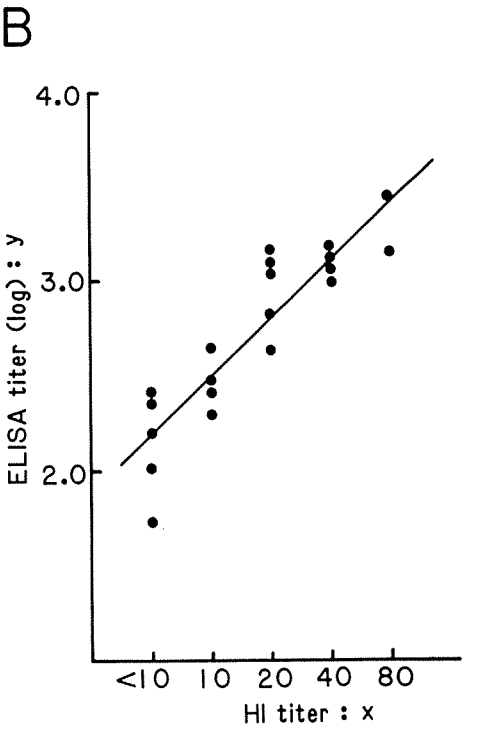
2. *Relationships of ELISA, N and HI titers in Serum II*

Serum samples (Serum II) from 20 children who had received two shots of JE vaccine with an interval of one month between shots were obtained 1–3 months after the second vaccination and investigated by the ELISA, and N and HI tests (Fig. 2). All but one serum sample showed positive titers by the ELISA and N test. However, five of the 20 serum samples showed negative titers in the HI test. The one exceptional serum sample showed negative titers in all three tests. A high correlation was observed between the ELISA and HI titers [correlation coefficient (r)=0.90; regression equation, $y=0.31X+2.20$; $X=\log_2 x/5$ (Fig. 2B)]. Definite, but less significant, correlations were also observed between the ELISA and N titers [$r=0.69$; $y=0.80x-0.30$ (Fig. 2A)], and between the N and HI titers [$r=0.58$; $y=0.23X+1.48$; $X=\log_2 x/5$ (Fig. 2C)].

3. *Relationships of ELISA, N and HI titers in Serum III*

Serum samples (Serum III) from 12 children who had received JE vaccine in the previous year and another booster dose in the second year were obtained 1–3 months after the booster dose and examined by the ELISA, and N and HI tests (Fig. 3). All the serum samples showed positive titers in all three tests. Moreover, their titers were considerably higher than those of Serum II. High correlations between the ELISA and N titers [$r=0.96$;





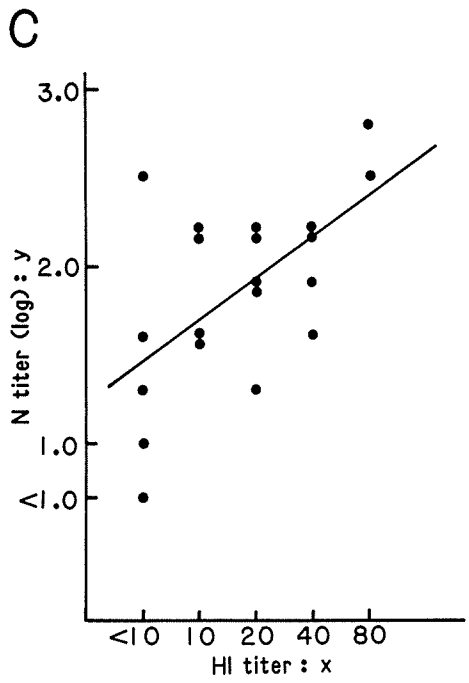


FIGURE 2. Relation of ELISA, N and HI titers in serum samples from 20 children who received two shots of JE vaccine in the first year. The correlations between ELISA and N titers ($r=0.69$, $y=0.80X-0.30$) (A), ELISA and HI titers ($r=0.90$, $y=0.31X+2.20$; $X=\log_2 x/5$) (B) and N and HI titers ($r=0.58$, $y=0.23X+1.48$; $X=\log_2 x/5$) (C) are shown.

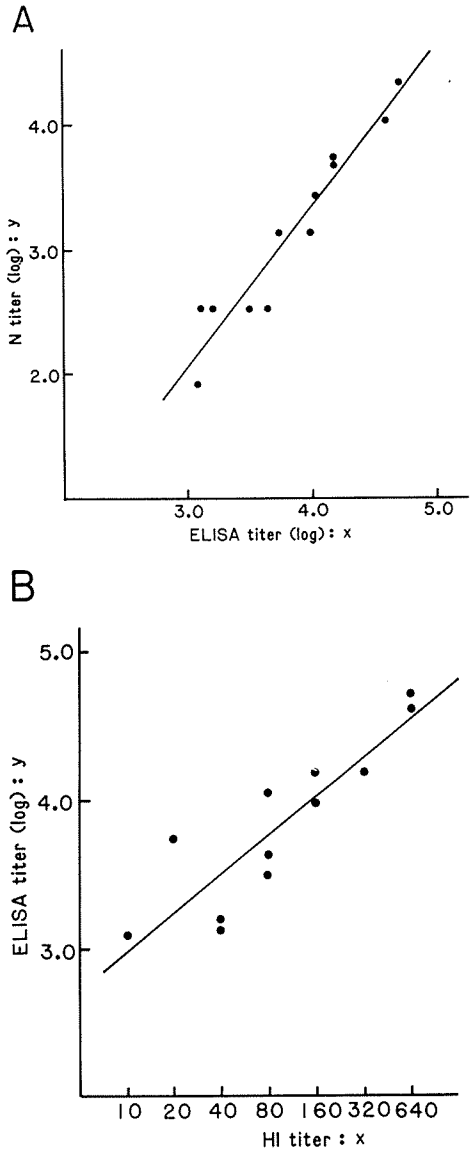
$y=1.29x+1.87$ (Fig. 3A)], between the ELISA and HI titers [$r=0.89$; $y=0.26X+2.72$; $X=\log_2 x/5$ (Fig. 3B)] and between the N and HI titers [$r=0.86$; $y=0.34X+1.67$; $X=\log_2 x/5$ (Fig. 3C)] were observed.

4. *Geometrical mean titers (GMT) of Serum II and Serum III*

Table 1 shows the GMTs measured by ELISA, and N and HI tests in Serum II and Serum III. The ELISA titers were considerably higher than the N and HI titers in both Serum II and III. After vaccination in the second year (Serum III), all ELISA, N and HI titers were markedly increased with ratios (Serum III/Serum II) of 13.1, 17.6 and 7.2, respectively.

DISCUSSION

ELISA has been widely used for serodiagnosis of viral infection. Igarashi et al. (1981) established a system for ELISA of JE virus,



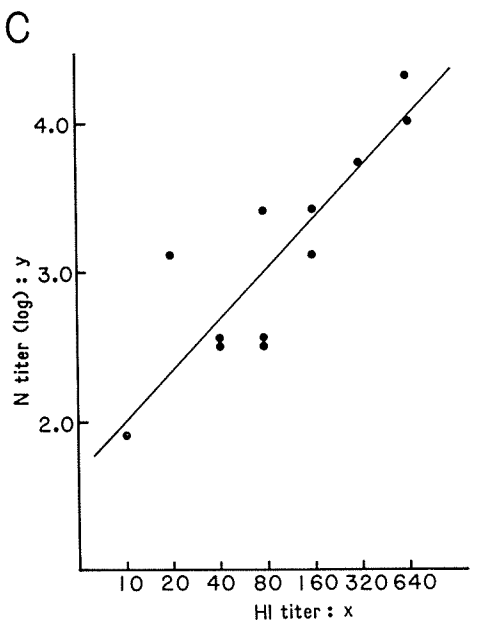


FIGURE 3. Relation of ELISA, N and HI titers in serum samples from 12 children who had received JE vaccine in the previous year and a booster dose in the second year. The correlations between ELISA and N titers ($r=0.96$, $y=1.29X+1.87$) (A), ELISA and HI titers ($r=0.89$, $y=0.26X+2.27$; $X=\log_2 x/5$) (B) and N and HI titers ($r=0.86$, $y=0.34X+1.67$; $X=\log_2 x/5$) (C) are shown.

and used it for detection of serum antibodies in humans (Bundo et al., 1981, 1982a) and animals (Bundo et al., 1982b). In the present study, we used this ELISA for measuring the antibody titers in children who had been immunized with the current JE vaccine.

We have found that many serum samples showed negative titers in the HI test, but high titers in the N test (unpublished data). Similarly in the present study, five of 20 serum samples (Serum II) showed negative titers in the HI test, but only one of them showed a negative titers in the ELISA and N test (Fig. 2). Low correlations were found between the ELISA and N titers ($r=0.69$), and between N and HI titers ($r=0.58$) in Serum II, but the correlation of their ELISA and HI titers was high ($r=0.90$). The results suggest that the antibodies detected in the ELISA and HI test recognized the same antigenic site on the virus.

The results in this study are consistent with the report of Bundo et al. (1983) that the ELISA titers are markedly increased by a booster immunization. After the booster injection we also observed increases in the antibody titers in the N and HI tests (Table 1), and high correlations of the antibody titers in the three tests (Fig. 3).

Since the ELISA was shown to have the same sensitivity as the N test (i.e., serum samples with positive N titers are also positive by the ELISA) ELISA can be used in place of the N test. Large scale serological screening for JE virus will be possible by the ELISA.

TABLE 1. *Geometrical mean titers (GMT) of Serum II and Serum III measured by ELISA, N and HI tests*

Test	GMT		Ratio (Serum III/ Serum II)
	Serum II	Serum III	
ELISA	520	6800	13.1
N	74	1300	17.6
HI	13	94	7.2

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