# Antibody Response in Japanese Encephalitis and Dengue Hemorrhagic Fever Patients Measured by Indirect ELISA

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Abstract: Antibody responses in sera from Japanese encephalitis (JE) and dengue hemorrhagic fever (DHF) cases were measured by the indirect micro ELISA using JE and dengue type 1 (D1) antigens. The responses of JE cases in Japan and primary encephalitis cases in Thailand were rather monospecific to JE antigen, in contrast to DHF cases whose antibody responses were cross-reactive to JE and D1 antigens even in the primary infection.

Key words: IgG-ELISA, Japanese encephalitis, dengue hemorrhagic fever

## INTRODUCTION

Japanese encephalitis (JE) has been prevalent in many countries in the East, Southeast to South Asia affecting numbers of cases with high mortality and grave sequelae (Miles, 1960; Umenai *et al.*, 1985). While large numbers of children in the Southeast

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Asia have been affected with dengue hemorrhagic fever (DHF) every year since its outbreak in the Philippines in 1953 (World Health Organization, 1966; Halstead, 1980a). Hemagglutination-inhibition (HI) test (Clarke & Casals, 1958) has widely been used for laboratory diagnosis on these diseases, however, the result was not always clear-cut especially in areas where both diseases are prevalent. The ambiguity is primarily due to the cross-reactions between JE and dengue viruses, both belonging to the family Flaviviridae sharing common antigens (Clarke & Casals, 1964; Westaway et al., 1985). Since ELISA was introduced as a new serological method with various advantages (Engvall & Perlman, 1971), the test has been applied to various viral and other infectious diseases (Voller et al., 1976; Sever & Madden, 1977). In the past several years, we have been applying the ELISA to the serodiagnosis and seroepidemiological survey on JE and DHF (Igarashi et al., 1981; Bundo et al., 1981, 1982a, b, 1983a, b; Morita et al., 1982; Fujita et al., 1983, Fukunaga et al., 1983; Bundo & Igarashi, 1983, 1985; Chanyasanha et al., 1984a, b; May La Linn et al., 1985; Igarashi, 1986). Through these studies, we showed that IgM-ELISA was useful for serodiagnosis, especially to differentiate JE and DHF, because of the higher specificity of IgM than IgG-antibodies (Westaway, 1968a, b; Edelman & Pariyanonda, 1973; Westaway et al., 1975), while IgG-ELISA appeared to be useful for measuring flavivirus antibody levels in seroepidemiological survey. When we examined IgGantibody levels among healthy inhabitants in Japan and Thailand by indirect micro-ELISA using JE and D1 antigens, Japanese people generally showed significantly lower titer to dengue type 1 (D1) antigen compared with the titer to JE, while Thai people showed almost similar titers to both antigens (Chanyasanha et al., 1984a). At first the result was considered to reflect multiple flavivirus infections (JE and dengue) in Thailand in contrast to Japan where JE is essentially the only flavivirus infection. Careful examination on JE cases in Japan compared with encephalitis and DHF in Thailand, however, revealed that JE and dengue virus infections, even in the primary cases, exhibited different antibody response to these 2 assay antigens as measured by the IgG-ELISA, and the results of the study are shown in this report.

### MATERIALS AND METHODS

Serum specimens: Paired sera from 33 DHF and 19 encephalitis cases in Chiang Mai, Thaiand (Igarashi et al., 1983), 11 DHF cases in Chanthaburi, Thailand (Okuno et al., 1980), 18 primary DHF cases in Rangoon, Burma (May La Linn et al., 1985), and 42 JE cases in Nagasaki, Japan (Bundo et al., 1981) were examined. According to the diagnostic criteria on DHF by the HI (World Health Organization, 1983), 8 of the 19 encephalitis and 4 of the 44 DHF cases in Thailand were categorized as primary infection, while 11 encephalitis and 40 DHF cases were secondary infection.

ELISA: Indirect micromethod of Voller et al. (1976) was slightly modified as described (Igarashi et al., 1981; Bundo et al., 1982a, b; Chanyasanha et al., 1984a). Formalin-inactivated and purified JE vaccine concentrate (Takaku et al., 1968) was used as JE antigen, while D1 antigen was prepared from culture fluid of Aedes albopictus clone  $C_{6/36}$  cells (Igarashi, 1978) infected with Hawaiian strain of D1 virus. The virion was concentrated and partially purified by polyetheylene glycol precipitation and ultracentrifugation (Bundo & Igarashi, 1983). Plastic U-shaped microplate (Immulon, Dynatech, USA) was coated with assay antigen diluted in coating buffer (1:80 for JE and 1:1600 for D1 antigens, respectively) and incubated at 4°C overnight. The plate was washed with PBS-Tween 3 times and reacted with test sera diluted 1:100 or 1:1000 in PBS-Tween. Standard positive serum of known end-point titer was serially diluted in 2-fold steps and reacted in parallel with test sera. After 1 hour's incubation at 37°C, the plate was washed and reacted with anti-human IgG or IgM goat IgG conjugated with horseradish peroxidase (HRPO) and diluted in PBS-Tween. The plate was incubated at 37°C for 1 hour followed by washing and peroxidase reaction with o-phenylenediamine and  $H_2O_2$ . The plate was incubated at room temprature for 1 hour in the dark and the reaction was stopped by adding 4 N  $H_2SO_4$ . Optical density of the colored product was measured at 490 nm in a Micro ELISA Autoreader (Dynatech, USA) with reference wavelength of 630 nm. The titer of test specimen was estimated by comparing its ELISA-OD with those of the serially diluted standard serum using a computer system (Morita et al., 1982).

Reagents: HRPO-conjugated anti-human IgG (heavy and light chain-specific) goat IgG and HRPO-conjugated anti-human IgM ( $\mu$ -chain specific) were obtained from Cappel Laboratories, USA. JE antigen was generously supplied from Kanonji Institute, Research Foundation for Microbial Diseases of Osaka University. *o*-Phenylenediamine dihydrochloride was the product of Wako Pure Chemicals, Co., Osaka.

#### RESULTS

Fig. 1 shows changes in IgG- and IgM-ELISA titers in paired sera of JE patients in Japan as measured by JE antigen. Out of 42 pairs, 18 pairs by IgM-ELISA and 25 pairs by IgG-ELISA showed 4-fold or more titer increase when measured by JE antigen. In contrast, Only 3 and 2 pairs showed significant titer increase by IgM- and IgG-ELISA, respectively, as measured by D1 antigen (Fig. 2).

Sera from encephalitis patients in Thailand were similarly tested and the results were shown in Figs. 3 and 4. The sepcimen were categorized into primary and secondary according to their HI antibody response. By JE antigen, 4 out of 6 primary and one out of 6 secondary encephalitis cases showed 4-fold or more titer rise in their paired sera by the IgM-ELISA, while 4 primary and 4 secondary cases showed significant titer rise by the IgG-ELISA (Fig. 3). When measured by D1 antigen, on the other hand, none of the case showed significant titer rise by the IgM-ELISA, however, IgG-ELISA detected 4 secondary encephalitis cases with significant titer rise (Fig. 4). Similar examination was performed on DHF cases from Thailand and the results are shown in Figs. 5 and 6. IgM-ELISA with JE antigen detected only a single primary case with 4-fold or more titer increase, in contrast to IgG-ELISA detecting 3 primary and 20 secondary cases with significant titer increase (Fig. 5). Almost similar rates of significant titer increase were obtained with D1 antigen, detecting 2 primary and one secondary cases by the IgM-ELISA and 2 primary and 19 secondary cases by the IgG-ELISA, respectively.

The above results were summarized into Fig. 7. showing frequency distribution of IgG-ELISA titers against JE and D1 antigens obtained for JE patients in Japan, primary and secondary encephalitis as well as DHF patients in Thailand. In this figure, the higher titer of either acute or convalescent serum specimen was used. JE patients in Japan showed higher titer against JE (titer range under 8000 with modal titer under 500). Similar tendency was observed with primary encephalitis in Thailand (most of the titers were in the range of 2000-16000 against JE and all were under 500 against D1). However, secondary encephalities as well as primary and secondary DHF in Thailand did not show significant difference in their titers measured by these 2 assay antigens. An interestion find-



Fig. 1. Changes in IgG- and IgM-ELISA titers in paired sera of JE patients in Japan as measured by JE antigen. Open circles represent IgM and closed circles IgG-ELISA antibody titers, respectively.



Fig. 2. Changes in IgG- and IgM-ELISA titers in paired sera of JE patients in Japan as measured by D1 antigen. Symbols are the same as in the legend to the Fig. 1.



ELISA titer of the first serum , in Log

Fig. 3. Changes in IgG- and IgM-ELISA titers in paired sera of encephalitis patients in Thailand as measured by JE antigen. Open markes represent IgM-and closed marks IgG-ELISA antibody titers, while triangles represent [primary and circles secondary encephalitis cases, respectively.



ELISA titer of the second serum, in Log

Fig. 4. Changes in IgG- and IgM-ELISA titers in paired sera of encephalitis patients in Thailand as measured by D1 antigen. Symbols are the same as in the legend to the Fig. 3.



Fig. 5. Changes in IgG- and IgM-ELISA titers in paired sera of DHF patients in Thailand as measured by JE antigen. Open marks represent IgM and closed marks IgG-ELISA antibody titers, while triangles represent primary and circles seondary DHF cases, respectively.



Fig. 6. Changes in IgG- and IgM-ELISA titer in paired sera of DHF patients in Thailand as measured by D1 antigen. Symbols are the same as in the legend to the Fig. 5.



Fig. 7. Distribution of IgG-ELISA titers as measured by JE and D1 antigens on sera from JE patients in Japan, encephalitis and DHF patients in Thailand. Closed columns represent the number of patients assayed by JE antigen, and open columns D1 antigen, respectively.

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Patients in Thailand

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Fig. 9. Comparison of IgG-ELISA titers measures by JE and D1 antigens on each serum from primary DHF cases in Rangoon, Burma. Closed circles represent acute phase (S1) sera and open circles convalescent phase (S2) sera, respectively.

ing was that the primary DHF also showed similar titers against JE and D1, although their titers were under 8000 in contrast to the titers exceeding 16000 in the secondary DHF. As reported previously (Bundo *et al.*, 1982b), all the sera from healthy individuals in Hokkaido, JE-nonendemic northern island of Japan, showed low titer under 500 against both antigens.

Fig. 8 compares IgG-ELISA titers against JE and D1 antigens assayed for each of the patient's sera. Each dot represents the higher titer of either acute or convalescent serum from each individual. All but 3 JE cases in Japan showed 4-fold or more higher titer against JE than against D1 anitgen. Also, all of the 8 primary and only 3 of the 11 secondary encephalitis in Thailand showed 4-fold or more higher titer against JE than against D1 antigen. On the other hand, none of the DHF cases, primary or secondary, showed significantly higher titer against D1 than JE antigen. Three of the 36 secondary DHF cases showed 4-fold or more titer against JE than against D1 antigen.

Since only 4 primary DHF cases were examined, we were not confident enough at this moment to mention that the IgG-antibody responses of JE and DHF are different. Therefore, similar examination was performed on the IgG-ELISA antibody response of 18 primary DHF cases in Rangoon, Burma (May La Linn *et al.*, 1985). The result (Fig. 9) compares IgG-ELISA titers against JE and D1 antigens measured for both acute (S1) and convaescent (S2) sera. Except a single S1 and 5 S2 sera, almost 83% of the sera tested did not show significant difference between IgG-ELISA titers assayed by JE and D1 antigens.

#### DISCUSSION

In order to detect significant titer increase in paired sera, IgM-ELISA was not more efficient than the IgG-ELISA. This is probably due to the more rapid production of IgM than IgG antibodies.

Our data indicate that JE virus infection produced more JE-specific IgG antibodies in contrast to dengue virus infection which, even in the primary infection, produced more antibodies cross-reactive to both JE and D1 antigens. As we reported before (Chanyasanha *et al.*, 1984a), healthy inhabitants in JE-endemic area in Japan showed more JE-specific IgG-ELISA titers compared with those in Thailand showing similar titers against JE and D1 antigens. The result is compatible with the present findings of cross-reactive IgGantibody response in DHF in contrast to more type-specific response in JE, because both JE and DHF are prevalent in Thailand, while JE is virtually the only flavivirus infection in Japan.

The reason why these 2 flavivirus infections showed different IgG-antibody response is intriguing, since both JE and dengue viruses belong to the same taxonomic family Flaviviridae (Westaway et al., 1985) possessing similar structure of the virion. Trent (1977) reported 3 antigenic determinants or epitopes, that is, type-specific, subgroupspecific, and cross reactive, on the envelope glycoprotein (E) of flaviviruses. Topographical relationships of several epitopes on JE virus E protein were finely analyzed by Kimura-Kuroda and Yasui (1983) using monoclonal antibodies. If the distribution or the arrangement of these epitopes are similar in JE and dengue viruses, then observed difference in the antibody response in JE and DHF may be explained by the different efficiency of the patients to recognize type-specific and subgroup-specific versus cross-reactive epitopes in these 2 flavivirus infections, because JE and dengue viruses are classified under separate subgroup in Flaviviridae (Porterfield, 1980). Athough cross-reactive epitope(s) are present on JE virion and is accessible to antibodies made in DHF, the epitope(s) may not be highly immunogenic in JE virus infection. Low immunogenicity of the epitope(s) may be due to the low efficiency of antigen presentation by macrophage system or inefficient helper-T cells responsible for the epitope (Unanue, 1981). The phenomenon may be due to the instability of cross-reactive epitope(s) on JE virion. On the other hand, crossreactive epitope(s) on dengue virion appear to be recognized efficiently by the infected humans, producing high levels of cross-reactive antibodies which can bind not only to the epitopes on dengue but also on JE antigen. Probably, the cross-reactive antibodies in DHF were produced far in excess compared with type-specific and/or subgroup-specific

antibodies, because DHF patient's sera did not show significant difference in their IgG-ELISA titers against JE and D1 antigens. This phenomenon may be related with the proposed "second infection theory" for the pathogenesis of DHF (Russell, 1971; Halstead, 1980b). In order to clarify the phenomenon on the molecular bases, fine structure of the E protein should be analyzed not only by monoconal antibodies, but also from the sequence data of genome RNA. Base sequence analysis on JE and dengue viruses is now going on (Sumiyoshi *et al.*, 1986), which will give more affirmative answer to this question. Another approach should be undertaken from the immunological aspects to disclose different efficiency of each component in the immune network toward JE and dengue viruses.

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日本脳炎とデング出血熱患者の IgG-ELISA 抗体反応

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日本脳炎とデング出血熱患者血清の IgG-ELISA 抗体反応を微量間接 ELISA 法により日本脳 炎ウイルスとデングウイルス1型抗原を用いて測定した。日本の日本脳炎患者とタイ国の脳炎初 感染患者は日本脳炎抗原に対して特異的反応を示したが、デング出血熱患者は初感染の場合でも 日本脳炎とデング1型抗原の両方に対して交差反応性を示した.

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