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Enzyme-linked Immunosorbent Assay (ELISA) on Japanese Encephalitis Virus. II. Antibody levels in the patient sera

Keiko BUNDO, Sachiko MATSUO, Akira IGARASHI

Department of Virology, Institute for Tropical Medicine, Nagasaki University

Abstract: Indirect enzyme-linked immunosorbent assay (ELISA) was performed to measure antibody titers in sera from patients clinically diagnosed or suspected of Japanese encephalitis (JE). Correlation was observed between the hemagglutination-inhibition (HI) and the total immunoglobulin (T-Ig) ELISA titers against JE virus. Significant rise accompanied by high convalescent T-Ig titers was observed for many paired specimens serologically diagnosed as JE by the HI test, in contrast to those HI-negative specimens showing no significant rise with relatively low convalescent T-Ig ELISA titers. Sucrose gradient sedimentation analysis on some sera has indicated that the ELISA using specific anti-human IgG or IgM conjugated with peroxidase could measure the antibody titers in each class separately. Assay on the IgM antibody titers showed certain correlation between the decrease in the HI titers after 2-mercaptoethanol (2ME) treatment and the IgM ELISA titers. The IgM ELISA could detect IgM antibody in many specimens for which significant amount of 2ME-sensitive HI antibody was not observed. Presence of high levels of IgG antibody was found to interfere the IgM ELISA titers. However, presence of rheumatoid factor did not exert appreciable effects to give any false positive IgM antibody against JE virus. Analysis on the data indicated that IgM ELISA could detect the largest numbers of JE-positive cases compared with the HI or T-Ig ELISA. Based on these observations, a tentative procedure of serodiagnosis on JE by the ELISA was proposed.

INTRODUCTION

Serological reactions have been used for the diagnosis on viral infections (Jawetz *et al.*, 1978), including Japanese encephalitis (JE) using hemagglutination-inhibition (HI), complement-fixation (CF), and neutralization (N) tests (Oya, 1978). Recently, enzyme-linked immunosorbent assay (ELISA) as developed by Engvall and Perlman (1971) has been added to those classical methods as a newly developed immunoassay (Voller, *et al.*, 1976; Sever and Madden, 1977), and Miyata *et al.* (1981) applied the

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長崎大学熱帯医学研究所業績，第1,110号

method for the seroepidemiological surveillance on arbovirus infections in domestic animals. We tried to use the ELISA method for the serodiagnosis on JE and devised a simple and reproducible method as reported previously (Igarashi *et al.*, 1981). Using the method we quantitated the total immunoglobulin (T-Ig) and IgM antibody titers against JE virus in sera of patients clinically diagnosed or suspected of JE, and discussed the applicability of the ELISA method for serodiagnosis on human JE proposing a tentative procedure of performing the assays.

MATERIALS AND METHODS

Antigen: Formalin-inactivated and purified JE vaccine concentrate (Takaku *et al.*, 1968) was kindly supplied by the Kanonji Institute, Research Foundation for Microbial Diseases of Osaka University. The solution contained 2.8 mg of protein per ml as determined by the method of Lowry *et al.* (1951) using bovine serum albumin fraction V (Armour, Ill. USA) as a standard. The solution was diluted to contain 25 and 100 μg of protein/ml for the assay of T-Ig and IgM or IgG, respectively.

Test sera: Following human sera were examined for the antibody titers against JE virus; (1) 452 specimens from patients clinically diagnosed or suspected of JE during the year of 1965 to 1975, which have been kept frozen at $-30°C$ in this Institute. (2) 8 sera from JE patients in the year of 1978 were obtained from Nagasaki Prefectural Istitute of Public Health and Environmental Sciences. As negative controls, sera were obtained from inhabitants around Nairobi, Republic of Kenya, where JE is not endemic.

ELISA procedures: The method was slightly modified from those described by Voller *et al.* (1976) as reported before (Igarashi *et al.*, 1981). The plastic Immulon U-miroplate (Greiner Labortechnik) was obtained from Sanko Pure Chemicals Co. Tokyo. The wells on the plate were coated with the antigen diluted in 0.05 M cabonate-bicarbonate buffer, pH 9.6, in a refrigerator overnight using 100 μl of the solution per well. The antigen was removed and the plate was washed with PBS-T (0.05 % Tween 20 in phosphate-buffered saline, pH 7.2) 3 times for 3 minutes each. Test sera diluted 1 : 100 or 1 : 1000 in PBS-T were distributed in the wells using 100 μl/well. Standard positive and negative sera were serially diluted in 2-fold steps and introduced in separate wells as references. The plate was incubated at 37°C for 1 hour. The sera were removed and the plate was washed as before. Peroxidase-labeled anti-human immunoglobulins diluted in PBS-T was distributed in the wells (100 μl/well), and the plate was again incubated at 37°C for 1 hour. The labeled serum was removed and the plate was washed as before. Substrate solution containing 0.5 mg of *o*-phenylendiamine dihydrochloride per ml and 0.02% of H_2O_2 in 0.05 M citrate-phosphate buffer, pH 5.0, was distributed in each well (100 μl/well) and the reaction was proceeded in the dark at room temperature for 1 hour. The reaction was stopped by adding 4 N H_2SO_4 (75 μl/well) and the optical density (OD) at 500 nm was recorded *in situ* by a Corona 2-

wavelength spectrophotometer using 600 nm as a reference wavelength. ELISA titer of the test specimen was estimated by comparing its OD with those on the standard curve obtained from a serial dilution of a standard positive serum, which was run in parallel.

2-Mercaptoethanol (2ME) treatment, acetone extraction and HI test : Test sera were treated with 2ME in order to destroy IgM antibody (Caul *et al.*, 1974) as described (Oya, 1978) with slight modifications. Fifty microliter of the serum was mixed with 150 μl of 0.13 M 2ME in PBS and was incubated at 37°C for 1 hour. Control specimen of 50 μl was mixed with 150 μl of PBS and was run in parallel. The sera were then extracted twice with 2.5 ml of cold acetone to remove nonspecific inhibitors, and dried *in vacuo*, and then restored with 500 μl of 0.05 M borate buffer in 0.15 M NaCl, pH 9.0, (BS) to make 1 :10 diluted serum specimens. Then they were tested for the HI antibody titers as described by Clarke and Casals (1958) using 8 units of antigen extracted by sucrose-acetone method from mouse brains infected with the Nakayama strain of JE virus.

Sucrose gradient sedimentation of the serum specimens: Immunoglobulins of IgM and IgG classes were separated as described by Caul *et al.* (1974) or Vesikari and Vaheri (1968). One half ml of the serum specimen diluted 1 : 5 in PBS was layered on top of the gradient columns of 4.5 ml volume consisting of 10 to 40 % sucrose in PBS. The specimens were centrifuged in a Beckman model SW-50.1 rotor at 35,000 rpm for 16 hours at 4°C. Twelve fractions from each tube were collected from the bottom after the run. Presence of IgM or IgG was determined by single-radial immunodiffusion using MBL-plate obtained from Medical-Biological Institute (Nagoya).

Detection of rheumatoid factors (RF) : The reagents RA (KW) to detect RF were obtained from Kyowa Pharmaceutical Co. (Tokyo). Fifty microliter of the test serum was mixed with an equal volume of latex-globulin suspension. Specimens showing visible aggregates were considered as RF positive.

Absorption of IgG with *Staphylococcus aureus* (Cowan I) : Ten percent (v/v) suspension of the bacteria was obtained from Chemo- and Serum Therapy Institute (Kumamoto) as a product of Absorb G. A volume of 130 μl of the suspension was spun down at 3,000 rpm for 30 minutes and the supernatant was removed. To the sedimented bacteria, 400 μl of the serum specimen diluted 1 : 100 in PBS-T was added and the suspension was incubated at 37°C for 1 hour and then bacteria were spun down again at 3,000 rpm for 30 minutes. The supernatant was used as the treated serum removed of IgG (Skang and Tjoetta, 1974; Leinikki *et al.*, 1978; Roggendorf *et al.*, 1980).

Reagents: Peroxidase-labeled anti-human immunoglobulins (IgA+IgG+IgM heavy and light chains), anti-human IgG and anti-human IgM were obtained from Cappel Laboratories, Pa. USA. *o*-Phenylenediamine dihydrochloride was the product of Wako Pure Chemicals Co. Osaka. 2-Mercaptoethanol was the product of Merck Co. Darmstadt, FRG.

Statistical methods: The methods described by Snedecor (1952) were followed.

RESULTS

Comparison of the HI titer with total immunoglobulin (T-Ig) ELISA titer : T-Ig ELISA titer was obtained for 163 serum speciments at 1:100 or 1:1000 dilution using peroxidase-labeled anti-human immunoglobulins (IgA+IgG+IgM, heavy and light chains) at 1:400 dilution. Each specimen was assayed more than twice and the titer was expresses as the geometrical mean value (GM), as for the HI titers, and was shown in Fig. 1. Each dot represent each serum specimen and the T-Ig titer appears to be approximately 10 fold higher than that of the HI. Between the logarithm of the HI (X) and the logarithm of T-Ig ELISA (Y) titers, relationship was observed with correlation coefficient of 0.75 with probability of less than 0.1 percent, and a linear regression line was drawn with equation of $Y=0.59X+2.15$, showing relatively higher T-Ig ELISA value for those specimens with lower HI titers. The specimens with T-Ig ELISA titer over 10,000 possessed the HI titers over 320, and T-Ig ELISA over 32,000 the HI titer over 640, respectively.

Changes of the T-Ig ELISA titers in paired sera : Many of the serum specimens were taken from the same individual patient as pairs or sequentially. The changes in their T-Ig ELISA titers of the "acute" and "convalescent" specimens were examined as above and compared. Fig. 2 shows the results with 29 pairs of the specimens which showed 4-fold or more increase in their HI titers with maximum HI titer over 40. All these patients were considered either as definitely, probably, or possibly, infected with JE virus, according to their maximum HI titers, as described by Oya and Okuno (1972). All of them possessed the T-Ig ELISA titers over 1,000 in their convalescence. Twenty two out of the 29 pairs showed 4-fold or more increase in their T-Ig ELISA titers, and 5 pairs showed more than 2-fold increase. Two pairs, although they did not show more than 2-fold increase, their T-Ig ELISA titer was over 10,000. Fig. 3 shows similar comparison of the T-Ig ELISA titers of 47 pairs which did not show 4-fold or more increase in their HI titers with some HI titers over 10. These specimens could be classified either into definitely (7 specimens), probably (7 specimens), possibly (6 specimens) infected with JE virus based on their maximum HI titers according to the diagnostic criteria (Oya and Okuno, 1972). However, twenty seven paris with HI titers less than 160 were classified as inconclusive by the HI test. Three out of the 47 pairs showed 4-fold or more increase in their T-Ig ELISA titers, and 8 pairs more than 2-fold increase. In Fig. 4 are shown the T-Ig ELISA titers of 30 pairs which did not show any detectable HI antibody titers against JE by the HI test and thus considered as negative with JE virus infection (Oya and Okuno, 1972). All of the 30 paris did not show more than 4-fold increase with their maximum T-Ig ELISA titers less than 1,000.

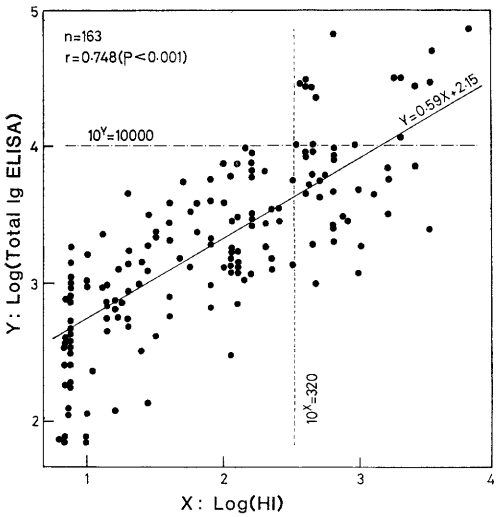

Fig. 1. Relationship between the HI and the total Ig ELISA titers. Antibody titers in 163 serum specimens were assayed by the HI and total Ig ELISA more than twice and their GM values in logarithm were plotted.

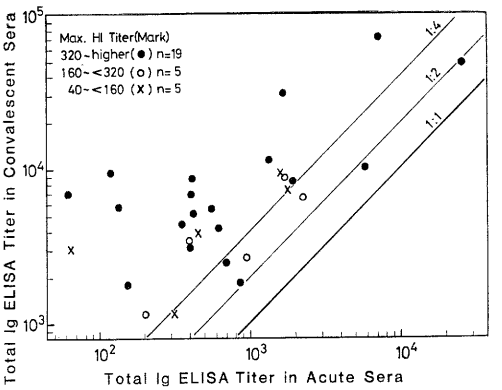

Fig. 2. Changes in the total Ig ELISA titers in paired sera with 4-fold or more rise in the HI titers. Each plot respresnts each of the 29 paired sera with their maximum HI titer over 320 (●), between 160 to 320 (o), and between 40 to 160 (x).

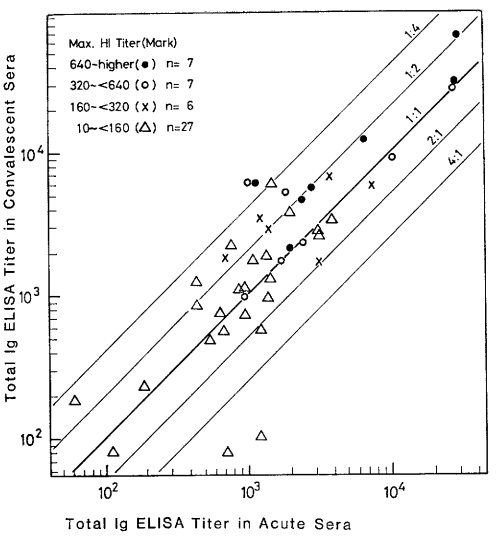

Fig. 3. Changes in the total Ig ELISA titers in paired sera without 4-fold or more rise with positive HI titers. Each plot represents each of the 47 paired sera with their maximum HI titer over 640 (●), between 320 to 640 (o), between 160 to 320 (x) and between 10 to 160 (△).

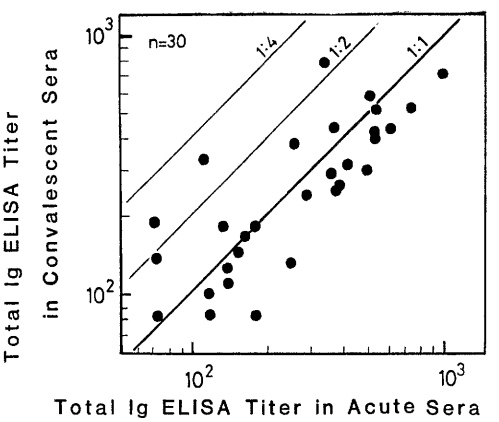

Fig. 4. Changes in the total Ig ELISA titers in paired sera without detectable HI antibody against JE antigen.

Assay on IgM antibody titers by the ELISA : The above mentioned results indicate that the informations obtained by assaying T-Ig ELISA titers did not tell significantly more than those obtained by the HI test. Since the presence of IgM antibody indicates recent exposure of given patients to the antigens used in the test, the determination of IgM antibody has some diagnostic significance (Jawetz *et al.*, 1978). In the case of JE, the existence of IgM antibody is demonstrated by examining the HI titers with and without 2ME treatment, taking 8-fold or more decresse in the titers by 2ME as the criteria (Oya, 1978). In order to test the validity of the assay system, 6 serum specimens with probable IgM antibody as revealed by the 2ME-sensitive HI antibody were fractionated by the sucrose gradient sedimentation as described in the Materials and Methods. Each fraction was assayed for the HI, T-Ig ELISA, IgG-ELISA, as well as IgM-ELISA titers with or without 2ME treatment. IgG- and IgM-ELISA titers were assayed by using gamma-chain-specific or mu-chain-specific anti-human immunoglobulin goat globulins labeled with peroxidase, at 1 : 200 and 1 : 100 dilutions, respectively. Fig. 5 shows the results with one of the 6 sera with IgM in and around fraction 5 and IgG fraction 8, respectively, as revealed by the single radial immunodiffusion (Panel C. D.) The HI titers as well as T-Ig ELISA titers distributed in 2 peaks in fractions 5 and 8, and the antibody activities in and around fraction 5 were destroyed by 2ME treatment, while those in and around fraction 8 were relatively resistant (Panel A and B). Assay on IgG ELISA titer and IgM ELISA titer demonstrated single peak of antibody activity in fraction 8 and in fraction 5, respectively, (Panel C and D), indicating that the assay using specific conjugated sera against IgG or IgM could assay the IgG or IgM antibody titers separately.

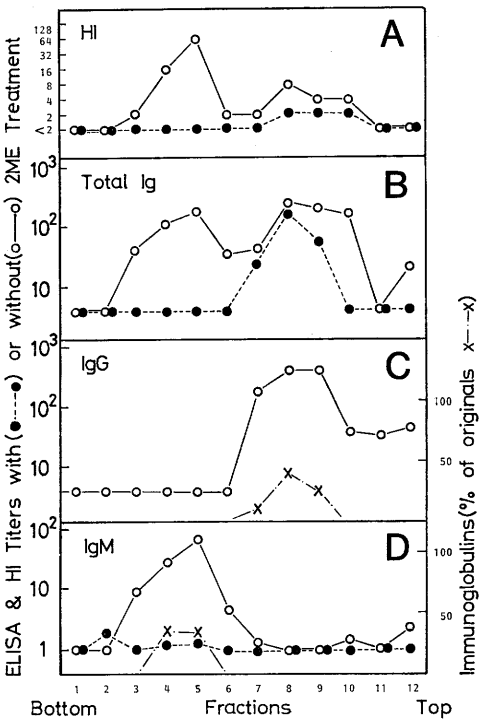

Fig. 5. Fractionation of a JE patient serum by sucrose gradient velocity sedimentation. A patient serum was diluted 1 : 5 in PBS and was fractionated as described in the Materials and Methods. Titers of the serum were HI: 453; T-Ig ELISA: 6139; IgG ELISA: 2289; IgM ELISA 617, which were reduced after 2ME to HI: 40; T-Ig: 880.

Comparison of the IgM-ELISA titer with 2ME-sensitive HI titer : Since above mentioned results supported the assay on IgM antibody by the ELISA, each of the 172 serum specimens was assayed for the HI and IgM ELISA titers with and without

2ME treatment. Fig. 6 shows the comparison between the 2ME-sensitive HI titer with the IgM ELISA titers. Between logarithm of the ratio of the HI titer without 2ME to that with 2ME treatment (X) and the logarithm of IgM ELISA titer (Y), relation was observed with correlation coefficient of 0.75 with probability less than 0.1 percent, and the regression line of the equation Y=0.93X +1.37. The specimens with the HI ratio (without to with 2ME) over 8 possessed the IgM ELISA titer over 54. There were 44 specimens with the HI ratio less than 8 and IgM ELISA titer over 54. These 44 specimens could further be classified as shown in Table 1. Twenty eight specimens showed the HI ratio (without to with 2ME) over 4, and they could probably possess the IgM antibody against JE virus, because the diagnostic criteria of HI ratio over 8 appears to be rather too strict to detect the IgM antibody by the HI test (Oya, personal communication). The 44 specimens could also be classified into those with low HI titer of less than 80 (18 specimens), with high HI titer over 400 (10 specimens) or intermediate (16 specimens). We could not detect the 2ME-sensitive HI antibody in the first and the second classes, because low HI in the control did not give the HI ratio over 8, and high IgG antibody will give a high background of 2ME-resistant HI titer. So that the direct assay on the IgM ELISA titer will detect more specimens possessing IgM antibody against JE virus.

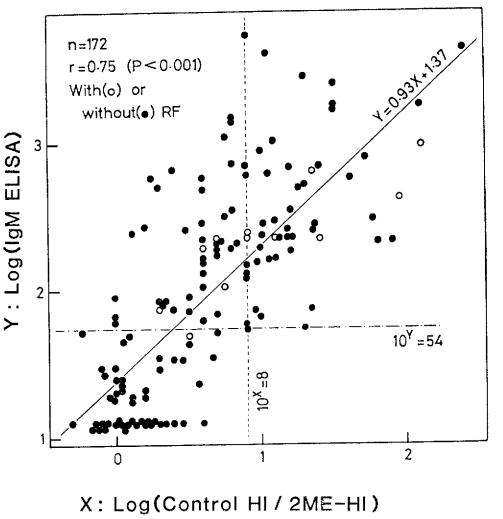

Fig. 6. Relationship between 2ME-sensitive HI antibody and IgM ELISA titers. Each plot represents each of 172 serum specimens, for which HI titers before and after the 2ME treatment and IgM ELISA titer were assayed, as shown by GM in logarithmic scale. Open circle represnts the specimens with detectable RF.

Table 1. Specimens with IgM-ELISA Titer over 54 and Ratio of 2ME-HI/Cont-HI under 8

Ratio of 2ME-HI/Cont-HI	No.	Control HI Titer		
		<80	80≦~<400	400≦
4≦~<8	28	9	13	6
<4	16	9	3	4
Total	44	18	16	10

Examination on the rheumatiod factor (RF) : Presence of the RF is reported to give a false positive results of the IgM antibody, because in the indirect ELISA assay RF will bind to anti-viral IgG antibody binding to the antigen (Salonen et al., 1980; Vejtorp, 1980). Absorption of IgG with protein A contained in *Staphylococcus aureus* (Cowan I) was reported to avoid such false positive IgM results (Leinikki et al., 1978; Roggendorf et al., 1980). One hundred and eighty serum specimens were treated with the method using Absorb G as described in the Materials and Methods and their IgM and IgG ELISA titers were examined before and after the treatment. IgG antibody was efficiently removed as determined by the IgG ELISA with average removal percent of 94.4±9.7 and average remainig titer of 213.5±378.1. Ratio of the IgM ELISA titer after Absorb G treatment to that of the control was compared with the T-Ig ELISA titer of the control for each serum specimen (Fig. 7). Specimens with open circle indicate the presence of RF, however, there was not significant trend showing that the IgM antibody titers of these specimens with RF decreased after Absorb G treatment. On the other hand, increase in the IgM antibody titers over 3-fold was observed with 6 specimens which possessed high T-Ig ELISA titer over 20,000. The result probably due to the competitive binding of the high amount of IgG antibody to the antigenic sites on the microtiter wells, pushing away the IgM antibody (Roggendorf et al., 1980). Several specimens showed significant decrease in their IgM ELISA titers abter Absorb G treatment, probably resulting from the absorption of IgM as well as IgG to the reagent, because *Staphylococcus aureus* protein A was sometimes reported to absorb IgM as well as IgG (Skang and Tjoetta, 1974. Roggerdort et al., 1980).

Interference on the IgM ELISA titer by IgG: Since some specimens with relatively high T-Ig ELISA titers showed significant increase in their IgM ELISA titers after Absord G treatment, indicating possible interference on the IgM assay by IgG antibody. examination was performed to see the effect of IgG fraction on the IgM titer after sucrose gradient sedimentation. Six serum specimens fractionated by the experiment described before were used in the test, and the result is shown in Table 2. Fraction 5 containing IgM from each gradient tube was mixed with equal or half of its volume of fraction 8 from the corresponding gradient tube and the final volume was adjusted to 100 μl with

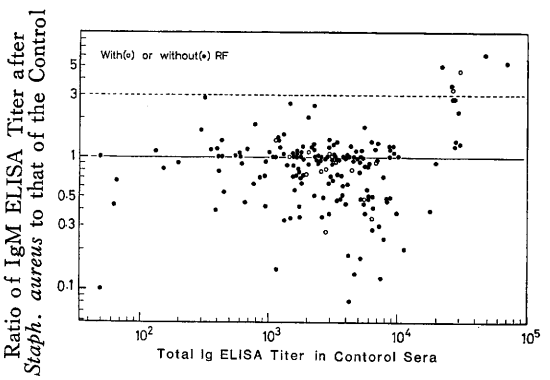

Fig. 7. Effect of *Staphylococcus aureus* (Cowan I) treatment on the IgM ELISA titer. Serum specimens were treated with Absord G (a product of *Staphylococcus aureus* (Cowan I)) followed by the IgM ELISA. Open circle represents the specimen for which RF was demonstrated.

Table 2. Interference on the IgM ELISA Titer by IgG Fractionated by Sucrose Gradiens

Assay mixture (μl)			IgG ELISA Titer (gradient tubes)					
Fr. 5	Fr. 8	PBS-T	(1)	(2)	(3)	(4)	(5)	(6)
50	0	50	9.6	11.0	2.8	9.3	10.0	8.4
50	25	25	9.2	12.5	0.5	10.5	14.0	8.4
50	50	0	10.5	12.0	0.3	10.5	9.4	8.8
IgG ELISA iter in Fr. 8			56.8	66.3	1260	67.0	54.3	47.5

PBS-T before assaying their IgM ELISA titers. In the case of gradient tube No. 3, significant interference on the IgM titer by the IgG fraction was observed, while no significant effect in other tubes. The result in tube 3 is probably due to the high IgG antibody titer as shown in Table 2. Possibility of low IgM titer in tube 3 giving such interference phenomenon could be excluded from the results using diluted IgM fraction from other tubes with relatively low IgG titers giving no significant interference. Since fraction 8 of tube 3 contained IgG titer of 1,260, 25 μl of the fraction 8 in 100 μl of the reaction mixture gives 1 : 4 dilution with final IgG titer of 315. This level of IgG roughly corresponds to those in the specimens which showed significant increase in their IgM ELISA titers after Absorb G treatment, because these specimens were diluted 1 : 100 before assay and their final T-Ig titers were thus around 300 after dilution. These results indicate the necessity of treating test specimens with *Staphylococcus aureus* protein A products in order to avoid the interference by high levels of IgG (probably over 20,000) on IgM ELISA titers.

Summary of the assay: Present data of ELISA on anti-JE antibodies in patient sera were summarized in Tables 3 and 4, comparing the results with HI tests. Table 3 shows the results with 106 paired sera, which could be classified into definitely (++), probably (+), possible (±), infected with JE, with 30 negative (−) and 47 inconclusive cases according to the criteria by the National Institute of Health of Japan (Oya and Okuno, 1972). Fifteen out of the 19 (++) specimens showed 4-fold or more increase in their T-Ig ELISA titers, however, 3, 4, and 2 pairs from 5(+), 5(±), and 47 inconclusive pairs also showed similar titer rise. On the other hand, all the 30 negative pairs showed T-Ig titers less than 1,000 with no significant increase in their titers. Examination on the IgM antibody by the HI test with 2ME treatment detected 27 cases including 13 from inconclusive cases. On the other hand, IgM ELISA detected 50 positive pairs if we take the borderline titer of 54. This number of positives could be increased to 54 when the specimens were treated with Absorb G to remove possible interference by high levels of IgG antibody. If all of these 54 cases really represent recent infection with JE virus, with no false positive results, IgM ELISA combined

Table 3. Serodiagnosis on JE by HI and ELISA (Paired sera)

HI test			No. of cases	Total Ig ELISA titer			IgM antibody	
Titer		Judgement on JE		4-fold or more rise (A)	Other than A & C	$<10^3$ throughout (C)	HI Cont/2ME $\geqq 8$	IgM ELISA $\geqq 54$
Rise	Maximum		106	24	42	40	27	50(+4)
$\geqq$ 4-fold	$\geqq 320$	++Definitely	19	15	4	0	12	17(+2)
	$160 \leqq$ <320	+ Probably	5	3	2	0	2	5
	$40 \leqq$ <160	± Possibly	5	4	1	0	0	5
Other than ++, +, ±. −		Inconclusive	47	3	35	10	13	21(+2)
<10 throughout		− Negatve	30	0	0	30	0	2
IgM antibody test	HI titer (Cont/2ME) $\geqq 8$		27	12	14	1	( ) after *Staph. aureus* Cowan I	
	IgM ELISA titer $\geqq 54$		50(+4)	23(+1)	24(+3)	3		

Table 4. Serodiagnosis on JE by HI and ELISA (Single serum)

HI test		No. of cases	Total Ig ELISA titer			IgM antibody	
Titer	Judgement on JE		$\geqq 10^4$ (A)	Other than A & C	$<10^4$ (C)	HI Cont/2ME $\geqq 8$	IgM ELISA $\geqq 54$
		156	14	88	54	43	86
$\geqq 640$	++Definitely	25	7	18	0	17	23(+2)
$320 \leqq$ <640	+ Probably	20	7	12	1	9	14(+2)
$160 \leqq$ <320	± Possibly	16	0	16	0	8	16
$10 \leqq$ <160	Inconclusive	69	0	39	30	9	30(+1)
<10	− Negative	26	0	3	23	0	3
HI titer(Cont/2ME) $\geqq 8$		43	2	37	4	( ) after *Staph. aureus* Cowan I	
IgM ELISA titer $\geqq 54$		86	7(+4)	69	10(+1)		

with treatment with *Staphylococcus aureus* protein A will detect the largest numbers of JE infection compared with classical HI test or T-Ig ELISA, and all the 19 pairs definitely diagnosed as JE by the HI could also be diagnosed as JE by the IgM ELISA. Results with single serum specimens as shown in Table 4 also support this consideration, giving the largest numbers of positives without missing the cases definitely diagnosed as JE by the HI test.

DISCUSSION

Present data indicate that the IgM ELISA could detect the largest numbers of cases with possible recent JE infections. Detection of IgM antibody was considered to be significant for the early diagnosis of various infectious diaeases (Jawetz *et al.*,

1978). Indirect IgM ELISA has been applied in various viral diseases for this purpose (Prévot and Guesdon, 1977; Hofmann *et al.*, 1979; Ukkonen *et al.*, 1980; McLean *et al.*, 1980; Krishna *et al.*, 1980; Hacham *et al.*, 1980; Gallo *et al.*, 1981; Corthier and Franz, 1981). In this method, presence of RF sometimes gives false positive IgM antibody titers (Salonen *et al.*, 1980; Vejtorp, 1980). This problem could be overcome by treating the serum specimens with *Staphylococcus aureus* protein A to remove IgG (Leinikki *et al.*, 1978; Roggendorf *et al.*, 1980), or with IgG-coated latex particles to remove RF (Roggendorf *et al.*, 1980; Kryger *et al.*, 1981), or fractionating IgG from IgM by sucrose gradient sedimentation (Caul *et al.*, 1974; Vesikari and Vaheri, 1968) or by column chromatography (Johnson and Libby, 1980). In our study, treatment by *Staphylococcus aureus* protein A did not appear to decrease the observed IgM antibody titers especially from those serum specimens with RF. Rather, the treament did give some effects to remove the apparent interference on the IgM titer by high levels of IgG antibody. Recently new methods have been reported to measure IgM ELISA titers without pretreatment of the serum specimens in order to avoid the false positive results by RF. The methods utilized "catching" anti-IgM antibody to coat the microplate (Duermeyer and van der Veen, 1978; Duermeyer *et al.*, 1979; Diment and Chantler, 1981; Kryger *et al.*, 1981; van Loon *et al.*, 1981; Roggendorf *et al.*, 1981). Application of this method should also be considered in the case of JE.

Based on the present data we would like to propose a tentative procedur of sero-diagnosis on JE by the ELISA. (1) Test sera should be diluted 1 : 100 and 1 : 1000 and measure T-Ig ELISA titers. IgM ELISA titers should be assayed at 1 : 100 dilution of the sera. (2) Specimens showing high T-Ig (over 20,000) with low IgM (under 54) titers should be treated with *Staphylococcus aureus* protein A followed by the IgM assay. (3) Any specimens showing either of the following characters could be considered as positive with recent JE infection: (i) IgM ELISA titer over 54. (ii) T-Ig titer over 31,000. (iii) 4-fold or more rise in T-Ig titers in paired sera. These criteria is tentative at present and should wait for further criticism. Especially, the data should be obtained to measure the IgM antibody level in JE nonendemic areas and the duration of the IgM antibody levels.

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日本脳炎ウイルスに対する免疫酵素測定法（ELISA）．Ⅱ．ヒト患者血清中の抗体価

分藤桂子，松尾幸子，五十嵐　章
（長崎大学熱帯医学研究所ウイルス学部門）

臨床的に日本脳炎（ＪＥ）と診断またはＪＥを疑われたヒト患者血清中のＪＥウイルスに対する抗体価を免疫酵素測定法（ELISA）の間接法により測定した．血球凝集抑制（ＨＩ）抗体価と全免疫グロブリン（T-Ig）ELISA 抗体価との間には相関性が認められた．ＨＩにより血清学的にＪＥと診断された対血清の多くは有意の抗体価上昇を伴う高い回復期 T-Ig ELISA 抗体価を示したが，ＨＩ陰性の材料は有意の抗体価上昇を示さず回復期の T-Ig ELISA 抗体価も比較的低値であった．庶糖密度勾配遠心法により分画した患者血清の ELISA 結果から，ヒト IgG または IgM に特異的に結合するペルオキシダーゼ標識抗体を用いることによって IgG または IgM 抗体価を別々に ELISA により測定できることが判明した．２メルカプトエタノール（２ME）処理によるＨＩ抗体価の低下度と IgM ELISA 抗体価との間には相関性が認められた．２ＭＥ感受性ＨＩ抗体価が有意に認められない場合にも IgM ELISA 抗体が認められる材料が可成りの数存在した．高力価の IgG 抗体が存在すると IgM ELISA 抗体価の測定値が阻害された．リウマチ様因子が存在してもＪＥウイルスに対する IgM ELISA が疑陽性となる明らかな例は認められなかった．得られた結果の分析により IgM ELISA はＨＩや T-Ig ELISA に比べより多くのＪＥ陽性例を検出できることが判明した．これらの結果に基づいて ELISA によるＪＥ血清診断法を提案した．