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Enzyme-linked Immunosorbent Assay on Japanese Encephalitis Virus

V. Antibody levels among inhabitants in endemic and nonendemic areas

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Abstract: Antibody levels against Japanese encephalitis (JE) virus antigen were measured by enzyme-linked immunosorbent assay (ELISA) on healthy inhabitants in JE-endemic (Kumamoto Prefecture) and nonendemic (Hokkaido Prefecture and Republic of Kenya) areas. Total immunoglobulin (Ig-) ELISA titers in the endemic area were distributed in broad range, in contrast to that in the nonendemic areas, which appeared to have certain limit values. However, IgM-ELISA titers in healthy inhabitants were rather low and observed in relatively low percentage of the people even in endemic areas. Comparison of these titer distributions with those of JE patients clinically and serologically diagnosed as JE by the hemagglutination inhibition (HI) test gave certain diagnostic criteria by the ELISA for JE patients observed in Japan.

INTRODUCTION

We have been trying to apply the ELISA for serodiagnosis on JE in human patients as well as to seroepidemiological study on swine sera (Igarashi *et al.*, 1981; Bundo *et al.*, 1981; 1982). However, it is necessary to know the levels of antiviral antibody

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among inhabitants in endemic and nonendemic areas in order to set up certain diagnostic criteria for JE by the ELISA as described in the text book (Voller *et al.*, 1976). Since the last large epidemic of JE in 1966, steady and dramatic decrease in the number of JE patient was observed in Japan, and less than 100 cases were reported in each year since 1972 (Health and Welfare Statistical Association, 1975). Most of these apparent JE cases are now confined in Southwest part of Japan, that is in Kyushu island. Especially Kumamoto Prefecture is one of the most JE-prevalent Prefecture in Japan (Oya, personal communication). On the other hand the northern island, Hokkaido has been free from JE for more than several decades. Sera from inhabitants in highlands in the Republic of Kenya were also used as another example of JE-nonendemic areas. Results of the ELISA on these healthy inhabitants were compared with those of apparent JE patients and certain diagnostic criteria were worked out for the serodiagnosis on JE by the ELISA.

MATERIALS AND METHODS

Antigen: Formalin-inactivated and purified JE vaccine concentrate (Takaku et al., 1968) was used as the ELISA antigen as described before (Igarashi *et al.*, 1981).

Sera: Following sera were obtained from healthy inhabitants: 149 specimens from 16 to 37 years old inhabitants in Hokkaido Prefecture were bled during the year of 1981; 519 specimens from various age groups in Kumamoto Prefecture were bled between July and October in 1981; 79 specimens collected in 1980 around Nyeri, situated about 1500 meters above the sea level in the Republic of Kenya. Besides these specimens 28 serum specimens from 11 patients clinically diagnosed as JE in Kumamoto Prefecture in 1981 were tested. These results were compared with the results obtained for JE patients collected in Nagasaki Prefecture in the years from 1965 to 1978.

ELISA: Indirect micro ELISA was performed according to Voller *et al.* (1976) with modifications as described (Igarashi *et al.*, 1981; Bundo *et al.*, 1981). Test sera were diluted 1:100 or 1:1000 and the color reaction was compared with those developed by serial dilution of a standard positive serum in order to calculate the ELISA titers. These calculations were preveiously performed by the graphical method, however, a computer system was recently introduced for the calculation as described in a separate paper (Morita *et al.*, 1982).

Hemagglutination-inhibition (HI) test: The method of Clarke and Casals (1958) was used with application of microtiter system (Sever, 1962). The test was performed in the Chemo-sero-therapeutic Research Institute and in Kumamoto Prefectural Institute for Public Health and Environmental Sciences using the antigen of JaGAr-01 strain of JE virus.

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

Reagents: Peroxidase-labelled anti-human IgG (heavy and light chains) was used

to measure immunoglobumin (Ig-) ELISA titer, and peroxidase-labelled anti-human IgM (μ-chain specific) to IgM ELISA titer. These enzyme conjugates were obtained from Cappel Laboratories. Pa. USA. Formalin inactivated purified JE vaccine concentrate was kindly supplied by the Kanonji Institute, Research Foundation for Microbial Diseases of Osaka University. *o*-Phenylenediamine dihydrochloride was the product of Wako Pure Chemicals Co. Osaka.

RESULTS

Frequency distribution of anti-JE ELISA titers: Fig. 1 shows frequency distribution of anti-JE Ig- (Panels A through H) and IgM (Panels I through P) ELISA titers among JE patients and healthy inhabitants in endemic as well as in nonendemic areas. Results with "JE" patients in Nagasaki were obtained previously as described before (Bundo *et al.*, 1981) and were reproduced here according to their serodiagnosis by the HI test (Oya and Okuno, 1972). The ELISA titers for the patients with paired serum specimens were shown by their highest titer and the results with single serum specimens were omitted. Dashed column represents those patients with demonstrable IgM antibody by the HI test combined with 2-mercaptoethanol (2ME) treatment (Oya, 1978). All the 26 patients with serodiagnosis of "Definite" JE showed Ig-ELISA titer over 1600 (Panel A) and IgM ELISA titer over 100 (Panel I). For those specimens with serodiagnosis of "Probable" or "Possible" JE showed Ig-ELISA titer over 800, and their IgM ELISA titers were over 100 except 2 specimens with "Probable" JE serodiagnosis. These 2 specimens either possessed high titered Ig-ELISA (over 12800) or showed 4-fold or more rise in the Ig-ELISA titers (data not shown) and 2ME-sensitive HI antibody was demonstrated for the latter specimen. Except this specimen, all the patients with demonstrable 2ME-sensitive HI antibody also possessed IgM-ELISA titer over 100 (Panels I through L). Except 2 specimens with 2ME-sensitive HI antibody and IgM-ELISA titer over 100 or 400, all the other specimens with serodiagnosis of "Inconclusive" possessed IgM ELISA titer less than 100, so are the specimens with "Negative" serodiagnosis (Panels L and M). The upper limit of the Ig-ELISA titer for "Inconclusive" specimens was 6400 and that in "Negative" specimens was 1600.

In Panels F and N are shown the results with sera obtained from inhabitants in Kumamoto Prefecture, JE-endemic area. Although Ig-ELISA titer distributed in wide range from less than 100 up to over 12800 with modal titer between 800 and 1600, (Panel F), their IgM-ELISA titer distributed in lower range and there were only 6 specimens which possessed IgM ELISA titer over 100 (Panel N). In contrast, the results with Hokkaido sera showed that their Ig-ELISA titer distributed only in low titered range of less than 800 (Panel G) and their IgM ELISA titer was less than 50 (Panel O). In the case of high-land inhabitants in Kenya (Panels H and P), their Ig-ELISA titer distributed

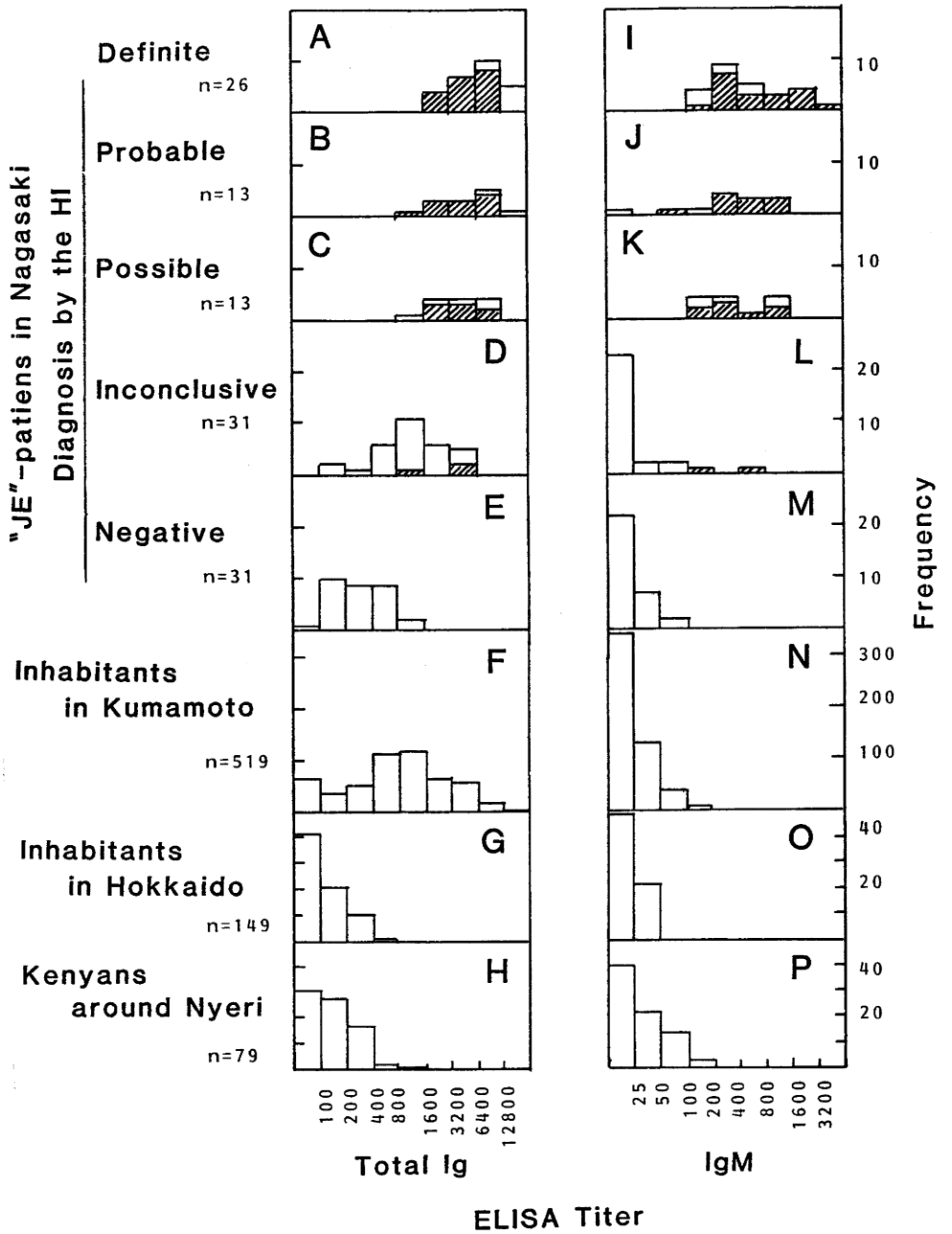

Fig. 1. Frequency distribution of ELISA titers among JE patients and among healthy inhabitants in JE-endemic and nonendemic areas. Dashed column represents the specimens for which 2ME-sensitive HI antibodies were demonstrated.

also in lower range although there were 3 specimens showing Ig-ELISA titer over 400 and IgM ELISA titer over 100. These specimens might represent some cross reactions with JE-related flaviviruses, such as West Nile or Zika virus.

Diagnostic criteria on JE by the the ELISA: Based on the above mentioned observations on the frequency distribution of ELISA titers and also on the observations on the changes of ELISA titers inpaired serum specimens (Bundo *et al.*, 1981), the specimens can be considered as positive with JE when

1) 4-fold or more rise in the ELISA titer was demonstrated in paired sera with Ig-ELISA titer over 800, or
2) IgM-ELISA titer over 100, or Ig-ELISA titer over 6400 is demonstrated in single serum specimen.

On the other hand, when Ig-ELISA titer is less than 800 throughout the course of the disease, JE can be excluded.

It may be noteworthy that 50 out of the 52 specimens with serodiagnosis of either "Definite", "Probable", or "Possible" JE by the HI test possessed IgM ELISA titer over 100, however, only 38 of the 52 specimens possessed 2ME-sensitive HI antibodies. Thus, IgM-ELISA could detect more specimens with probable recent infection than the HI test combined with 2ME-treatment.

Age distribution of antibody titers in endemic and nonendemic areas: The results of the HI and ELISA tests on healthy inhabitants in Kumamoto and Hokkaido Prefectures were rearranged according to the age groups. Tables 1, 2 and 3 represent the age distribution of the HI, Ig-ELISA, and IgM-ELISA titers in Kumamoto, respectively. In age group of 0-4 years old, many individuals did not have significant levels of antibodies against JE either by the HI or Ig-ELISA (Table 1 and 2). However, in other age groups over 5 years old, antibody titers distributed in wider range and 12 specimens

Table 1. Age distribution of HI titers among healthy inhabitants in Kumamoto Prefecture, 1981

Age group	HI titer									
	<10	10	20	40	80	160	320	640	Total	GMT
0– 4	54	1		2	1	3	1		62	7
5– 9			3	4	3	7	8	5	30	153
10–14			2	6	9	1			18	57
15–19		2	7	13	7	9	4		42	61
20–29	5	4	7	9	4	4	5	1	39	41
30–39	10	8	18	23	4	6	2	1	72	28
40–49	5	7	15	34	7	12	3	1	84	40
50–59	5	7	23	16	13	9	3	2	78	39
60–	9	8	11	24	21	15	4	2	94	46
Total	88	37	86	131	69	66	30	12	519	

Table 2. Age distribution of Ig-ELISA titers among healthy inhabitants in Kumamoto Prefecture, 1981

Age group	Ig-ELISA Titer									Total	GMT
	| 99	100 | 199	200 | 399	400 | 799	800 | 1599	1600 | 3199	3200 | 6399	6400 | 12799	12800 |		
0– 4	36	17	1	2		2	2	2		62	129
5– 9				6	5	12	3	3	1	30	1943
10–14			1	5	6	6				18	1109
15–19		1	1	7	15	9	8	1		42	1450
20–29	1	3	5	7	12	5	6			39	876
30–39	9	4	11	22	18	3	5			72	521
40–49	6	3	12	24	24	5	6	3	1	84	738
50–59	6	5	12	23	16	8	6	2		78	664
60–	7	4	8	17	20	14	19	5		94	1072
Total	65	37	51	113	116	64	55	16	2	519	

Table 3. Age distribution of IgM-ELISA titers among healthy inhabitants in Kumamoto Prefecture, 1981

Age group	IgM-ELISA titer				Total	GMT
	| 25	25 | 49	50 | 99	100 | 199		
0– 4	42	8	7	5	62	20
5– 9	4	13	13		30	39
10–14	12	5	1		18	17
15–19	11	23	8		42	27
20–29	19	18	2		39	21
30–39	58	12	2		72	15
40–49	66	15	3		84	15
50–59	61	16	1		78	15
60–	72	20	1	1	94	15
Total	345	130	38	6	519	

possessed HI titer of 640 and 18 specimens with Ig-ELISA titer over 6400, respectively. Geometrical mean titer (GMT) of the HI and Ig-ELISA in each age group is shown in Fig. 2. Fig. 3 shows the percent positive of the specimens in each age group either by the HI test (over 10) or by the Ig-ELISA (over 400). In age group of 0-4 years old, only 12.9 percent of the specimens possessed HI and/or Ig-ELISA antibodies, with GMT of 7 and 129 for the HI and Ig-ELISA, respectively. The positive percent rose up to 100 % in age group of 5-9 years old with GMT of 153 and 1943 by the HI and Ig-ELISA, respectively. The positive percent appears to remain at high level up to the age of 19, then slightly going down. This downward trend is slightly more remarkable

by the Ig-ELISA, compared with the HI test. This downward trend is also noticeable in GMT of the HI and Ig-ELISA titers in older age groups, however, the trend appeared to be parallel for the HI and the ELISA (Fig. 2). In the case of IgM-ELISA, however, titers distributed in lower ranges as shown in Fig. 1 already, and there were only 6 specimens out of 519 which showed IgM-ELISA titer over 100. Five of the 6 specimens were in the age group of 0-4 years old and another specimen in the age group over 60 years old (Table 3). Table 4 shows the results of Hokkaido sera. Since the specimens were taken mostly from young adult, they were classified into only 3 age groups. Compared with the data of the same age groups in Kumamoto, the Ig-ELISA titers in Hokkaido people are significantly less and distributed only in lower titer ranges, and so were the IgM titers also.

Correlation between the Ig-ELISA and the HI titers among healthy inhabitants in endemic areas: Table 5 shows the results of statistical rearrangement of the data with Kumamoto sera in order to see the correlation between the HI and the Ig-ELISA titers. Correlation was observed between the logarithm of the HI (X) and that of the Ig-ELISA (Y) with correlation coefficient of $r=0.85$ ($p<0.001$) with linear regression equation of $Y=0.88X+1.47$. The result is similar to those observed for JE patients in Nagasaki

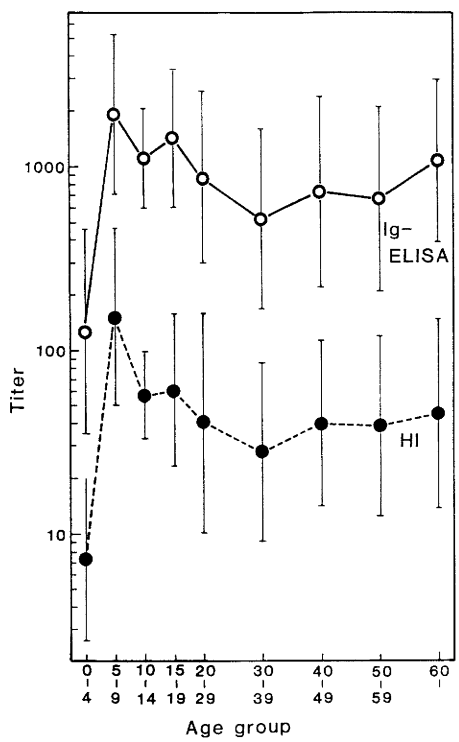

Fig. 2. Age distribution of the HI and Ig-ELISA titers among healthy inhabitants in Kumamoto Prefecture, 1981. Data in Tables 1 and 2 were rearranged with standard deviation as shown by the vertical lines, for the HI (●……●) and for the Ig-ELISA (○——○) titers.

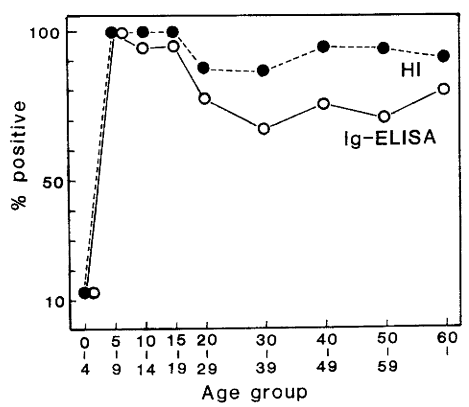

Fig. 3. Age distribution of antibody positive rate among healthy inhabitants in Kumamoto Prefecture, 1981. Positive rates were calculated for those having HI titer over 10 (●……●) or Ig-ELISA titer over 400 (○——○) in each age group.

Table 4. Age distribution of Ig- and IgM-ELISA titers among healthy inhabitants in Hokkaido Prefecture

Age group	Ig-ELISA titer						IgM-ELISA titer			
	–99	100–199	200–399	400–799	Total	GMT	–24	25–49	Total	GMT
15–19	39	22	11		72	86	45	27	72	17
20–29	44	20	10	1	75	84	60	15	75	15
30–39	1	1			2	74	1	1	2	22
Total	84	43	21	1	149	85	106	43	149	16

Table 5. Correlation between the HI and Ig-ELISA titers among healthy inhabitants in Kumamoto Prefecture, 1981

Ig-ELISA titer	HI titer									
	<10	10	20	40	80	160	320	640	Total	GMT
– 99	58	4	3						65	6
100–199	23	7	5	2					37	8
200–399	5	15	19	11	1				51	17
400–799	1	8	39	48	16	1			113	31
800–1599	1	2	18	53	25	16		1	116	50
1600–3199			2	14	12	21	14	1	64	116
3200–6399		1		2	11	22	14	5	55	173
6400–12799				1	4	6	1	4	16	182
12800–							1	1	2	453
Total	88	37	86	131	69	66	30	12	519	
GMT	88	290	489	867	1476	2624	3522	4831		

(Bundo *et al.*, 1981) and swine sera (Bundo *et al.*, 1982).

ELISA on JE patient sera collected in Kumamoto Prefecture: In 1981 14 JE patients were reported, and 13 of them were diagnosed as JE by the HI test or other criteria (Report from the Department of Health, Kumamoto Prefecture, 1981). Sera were taken from 11 of these serodiagnosed JE patients and were examined by the ELISA as shown in Table 6. All the 11 patients did not have the vaccination history against JE virus. Pationts No. 1 and 2 died early and did not give clear answer either by the HI or the ELISA. Patients No. 3, 6, and 7 showed 4 fold or more rise in their sera sequentially bled during the course of the disease both by the HI and by the ELISA, also their IgM ELISA titers were quite high and 2ME-sensitive HI antibodies were demonstrated for No. 3 and 7. Patients No. 4 and 5 showed 4 fold or more rise in the HI titer and more than 2-fold rise in Ig-ELISA titers. Because these specimens showed significantly

Table 6. Serological tests and serodiagnosis on JE patients reported in Kumamoto, 1981

No.	Age sex	Date of onset	outcome (date)	Sampling (days of disease)	HI test titer contr.	HI test titer 2ME	HI test diagnosis HI	HI test diagnosis 2ME	ELISA titer Ig	ELISA titer IgM	ELISA diagnosis Ig	ELISA diagnosis IgM
1	65F	Aug. 10	dead (Aug. 18)	6	80	40	?*	?	1632	29	?	?
2	68F	Aug. 17	dead (Aug. 20)	4	10	nt**	?	?	273	25	?	?
3	80F	Aug. 20	psychoneurologic disturbances	3	<10	nt			644	190		
				7	160	10	++	+	2985	946	+	+
				15	640	160			7341	1253		
				28	2560	160			4269	380		
4	71F	Aug. 24	recovered (Oct. 21)	5	2560	40	++	+	5449	1212		
				12	2560	320			10867	794	+	+
				30	2560	640			11763	527		
5	69M	Aug. 28	recovered (Sept. 30)	3	160	<10	++	+	2323	198	+	+
				9	640	40			5723	300		
				28	1280	320			6952	217		
6	62M	Aug. 27	recovered (Oct. 9)	5	20	<10	++	−	698	35	+	+
				13	320	320			6073	182		
7	5M	Aug. 30	psychoneorologic disturbances	6	<10	nt	++	+	155	12	+	+
				11	80	<10			1453	213		
				16	2560	20			45087	470		
8	79F	Aug. 23	neurological disturbances	37	113	10	?	+	1324	25	?	?
				51	160	20			1481	12		
9	23F	Aug. 23	psychonearologic disturbances	18	640	640	++	−	23812	30	+	?
				28	640	640			18476	30		
				44	320	320			15399	28		
				55	640	160			15277	27		
				60	320	320			13247	31		
				74	320	320			19071	34		
10	53F	Aug. 17	psychoneurologic disturbances	25	160	10	?	+	4008	365	?	+
				43	160	10			3023	145		
11	2M	Aug. 25	psychoneurologic disturbances	44	1280	160	++	+	27540	1045	+	+

*? : inconclusive **nt : not tested

high HI and Ig-ELISA titers, they can be considered as JE patients. Also their IgM ELISA titers were significantly high and both of them showed 2ME-sensitive HI antibodies. Sera from patient No. 9 were taken rather late after onset of the disease, thus both 2ME-sensitive HI antibodies and IgM-ELISA titer were not demonstrated, however, the titer by the HI and Ig-ELISA were high enough to diagnose the patient as JE.

The same conclusion can be drawn for the patient No. 11. Patient No. 10 did not have significantly high titered antibodies by the HI or Ig-ELISA, however because of 2ME-sensitive HI antibodies and IgM-ELISA, the patient can be diagnosed as JE. On the other hand, patient No. 8 showed 2ME-sensitive HI antibodies without significant amount of IgM-ELISA. This is the only one example which showed such a result. Treatment by the *Staphylococcus aureus* protein A did not give positive IgM ELISA for patient No. 9. Except patient No. 8, serodiagnosis by the ELISA according to our criteria could give similar results for those typical JE patients.

DISCUSSION

In Kumamoto Prefecture, JE-endemic area, 83 % of the specimens tested in this study possessed antibodies by the HI test, and their antibody distributed in wide range of the titers both by the HI and Ig-ELISA. However, in age group of 0-4 years old, antibody prevalence is at low level of 12.9 %. The figure rose up sharply to 100 % in age group of 5-9 years old. This might be due to the vaccination schedule which covered the age group of 3-15 years old with 85.5 % of the population in this age group. Also the antibody positive rate is partly due to the inapparent infection. The fact that several healthy people showed quite high antibody titers either by the HI or the Ig-ELISA (over 640, or 6400, respectively) may obscure the serodiagnosis by simple HI or Ig-ELISA on a single serum specimens, unless paired sera are tested or IgM antibodies were examined. Six of the 519 Kumamoto sera showing IgM-ELISA over 100 might be due to the result of inapparent infection, because 4 out of 5 of them, in age group of 0-4 years, did not have vaccination history. However, only one JE patient, No. 11, was classified in this age group. In contrast, 7 of the 11 patients were in the age group of more than 60 years old, and only one out of the 94 specimens of this age group of healthy people showed significantly high IgM antibodies by the ELISA. Since vaccines were given in young age groups, one of the 5 individuals with high IgM ELISA in age group of 0-4 years had the vaccination history. His IgM antibody may be due to the vaccination. This problem requires further studies to know the antibody response in the vacciness, especially in relation to the production of IgM antibodies. If we assume that all the IgM-ELISA titers over 100 are due to the inapparent infection, we may be able to guess that the ratio of apparent to inapparent infection of JE virus is age dependent, that is more apparent infection in older age group compared with young ones.

Frequency distribution of antibody titers among healthy individuals in JE-endemic and nonendemic areas and those in JE patient gave us certain criteria concerning serodiagnosis on JE by the ELISA. The criteria enable us to draw a conclusion even with a single serum specimen if IgM-ELISA is performed. The method is more easily be performed and may be more sensitive than the HI test combined with 2ME treatment, and will give future application to the clinical materials. The criteria should be used

only in those areas where JE virus is the only prevalent flavivirus. Cares should be taken and more strict criteria should be worked out in those areas where multiple flavivirus infections coexist, such as in Southeast Asia where dengue and JE viruses coexist.

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日本脳炎ウイルスに対する免疫酵素測定法（ELISA）．Ⅴ．流行地と非流行地における抗体価のレベル

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日本脳炎（JE）ウイルス抗原に対する抗体レベルを酵素免疫測定法（ELISA）により，JE 流行地（熊本県）と非流行地（北海道及びケニア共和国）の健康人について測定した．流行地における総免疫グロブリン（Ig-）ELISA 抗体価は広い範囲に分布したのに対して，非流行地においてはある限られた値を示した．しかし，健康人の IgM-ELISA 抗体価はむしろ低く，流行地の人においても少数にのみ認められた．臨床的に，また血球凝集抑制法（HI）により血清学的に JE と診断された JE 患者の抗体価の分布を健康人の抗体価分布と比較することにより，日本における JE 患者に対する，ELISA による診断基準が得られた．

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