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LABORATORY DIAGNOSIS OF JAPANESE ENCEPHALITIS COMPARISON OF THE FLUORESCENT ANTIBODY TECHNIQUE WITH VIRUS ISOLATION AND SEROLOGIC TESTS

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SUMMARY During 1965-1967, 194 cases who died with clinical manifestations of Japanese encephalitis (JE) were studied virologically by various laboratory tests, such as the direct fluorescent antibody test (FAT), virus isolation test and serologic tests. Of the cases 160 were confirmed by these tests and 122 of the latter 160 cases by the FAT alone.

The FAT is more sensitive and quicker for diagnosis of JE than other virological tests and could be used within 28 days from the onset of the disease, and especially in the early stage in all but one case. The FAT provided a positive diagnosis in 82 cases that were not confirmed by virus isolation and in 88 cases not proved by serologic tests. The FAT also demonstrated the JE virus antigen in the nerve cells of the pons area only of an extremely protracted case (151 days after the onset) in which the antibody titer was high from the 7th to 126th day after the onset. However, no virus could be isolated from the brain tissues of this patient.

A brain puncture was tested of obtaining brain specimens with an instrument after biopsy and these specimens were used for tests by the FAT and virus isolation. This method proved convenient and was easier than autopsy for rapid diagnosis of JE.

In 1967, no viremia was detected by virus isolation in 64 patients within 10 days from the onset of the disease, while their neutralizing antibody titers had already reached 1:100-1:10,000.

INTRODUCTION

Since 1924, epidemics of JE have occurred annually in Japan. Recently, the main distribution of the epidemics has shifted to western

districts of Japan including Osaka prefecture (OYA, 1967). In Osaka from 1965, epidemiological and ecological studies on JE have been

organized and there has also been good progress in the diagnosis of patients with this disease.

Some cases could not be confirmed virologically due to rapid death from JE before there was a significant antibody response (TOTANI, 1967). Brain specimens were seldom obtained from them for the FAT. This has resulted in somewhat uncertain results in the epidemiological survey of JE in Osaka. In Osaka, 344 cases who died with clinical manifestations of JE were reported in epidemics of JE in 1965-1967. Among 194 brain specimens from these cases, 28 were taken by autopsy and 166 by brain puncture (ISHII, 1963). Attempts to clarify the undetermined cases by serologic tests were made by the FAT (KUSANO *et al.*, 1966) for a full understanding of the epidemic in Osaka.

This paper presents results obtained by the FAT as compared with those by virus isolation and serologic tests in these 194 cases including an extremely protracted case. The importance

of the FAT in the epidemiological survey of JE is also reported.

MATERIALS AND METHODS

1. Sources of specimens

Specimens for the FAT and virus isolation were brain tissues from 194 cases who were suspected to have died of JE by clinical diagnosis in 1965-1967 (Table 2). Serologic tests were carried out on these cases every other week after admission. In 1967 blood specimens were also obtained from 64 inpatients suspected to have JE by clinical diagnosis, every other day within 10 days after the onset of the disease. Virus isolation studies were done on all specimens and the neutralization test (NT) on the first specimens taken (Table 8).

2. Fluorescent antibody technique

1) Method of obtaining brain specimens

The specimens were taken as soon after death as possible.



FIGURE 1 Instrument Used for Obtaining Brain Specimens.

a. Autopsy

In all 25 cases (Table 5), the cornu ammonis, substantia nigra, thalamus and cortex cerebri which showed typical histopathological lesions of JE (SHIRAKI, 1967) were collected.

b. Brain puncture

The instrument (Fig. 1) for brain puncture was inserted toward the thalamus about 3 cm from the base of the cranium at an angle of 45° by the nasal route.

2) Preparation of frozen sections of brain specimens

The specimens obtained were immediately frozen by dipping them into n-hexane at -70°C. The specimens were sliced into 4 μ thick sections in a cryostat (SLEE Co.), and were immediately treated with acetone for 5 min. at room temperature (in 1965) or with carbon tetrachloride for 20 min. at room temperature (in 1966-1967) (AOYAMA, 1967). Other specimens taken were immediately stored at -70°C until they were examined.

3) Preparation of fluorescein-labeled antibody solution

a. Preparation of antiserum

Sera of pigs which were naturally infected with JE (in 1965-1966) and serum from a patient with JE (in 1967) were used. These showed high hemagglutination-inhibition (HI) antibody titers of 1: \geq 2560 and 1:10,000, respectively, against the Nakayama strain of JE virus.

b. Conjugation

The serum γ -globulin fraction was conjugated by the method of MARSHALL *et al.* (1958) and GOLDSTEIN *et al.* (1961). The conjugates in 1965 and 1966-1967 were purified by the method of KAWAMURA (1966) and McDEVITT *et al.* (1963), respectively. All procedures were performed at 4°C. The fluorescein

isothiocyanate/protein ratio of the conjugates was 2.5-4.5 $\times 10^{-3}$.

c. Absorption of conjugates

Labeled sera were absorbed with normal human brain and liver acetone powder by COONS' method (1955).

4) Reaction of conjugates with antigen in brain sections of suckling mice infected with either JE, Dengue type 2, Mayaro, Sindbis or Semliki forest virus

The absorbed conjugates did not show any cross-reaction with group A arbovirus, but reacted strongly with Dengue type 2 of group B. The maximum titer of conjugates giving good staining were 1:128 against JE virus.

5) Staining

Sections were overlayed with drops of the conjugates and kept at 4°C overnight in a moist chamber and then washed 3 times with phosphate buffered saline (PBS).

6) Observation

Stained specimens were observed by fluorescent microscopy (Nikon Optical Co.). Specimens were regarded as "positive" when they contained a nerve cell which fluoresced due to the specific antigen-antibody reaction (Fig. 2a and 2b).

3. Isolation of JE virus from brain and blood

In 1965 and 1966, a dose of 0.02 ml of 20 per cent brain suspension in M/100 PBS (pH: 7.2) containing 2 per cent chicken serum was inoculated intracerebrally into a litter of ddO mice within 5 days after birth. In 1967, a dose of 0.2 ml of the same brain suspension or heparinized blood was injected intraperitoneally into one litter of ICR mice within 3 days after birth. When abnormal signs were observed in

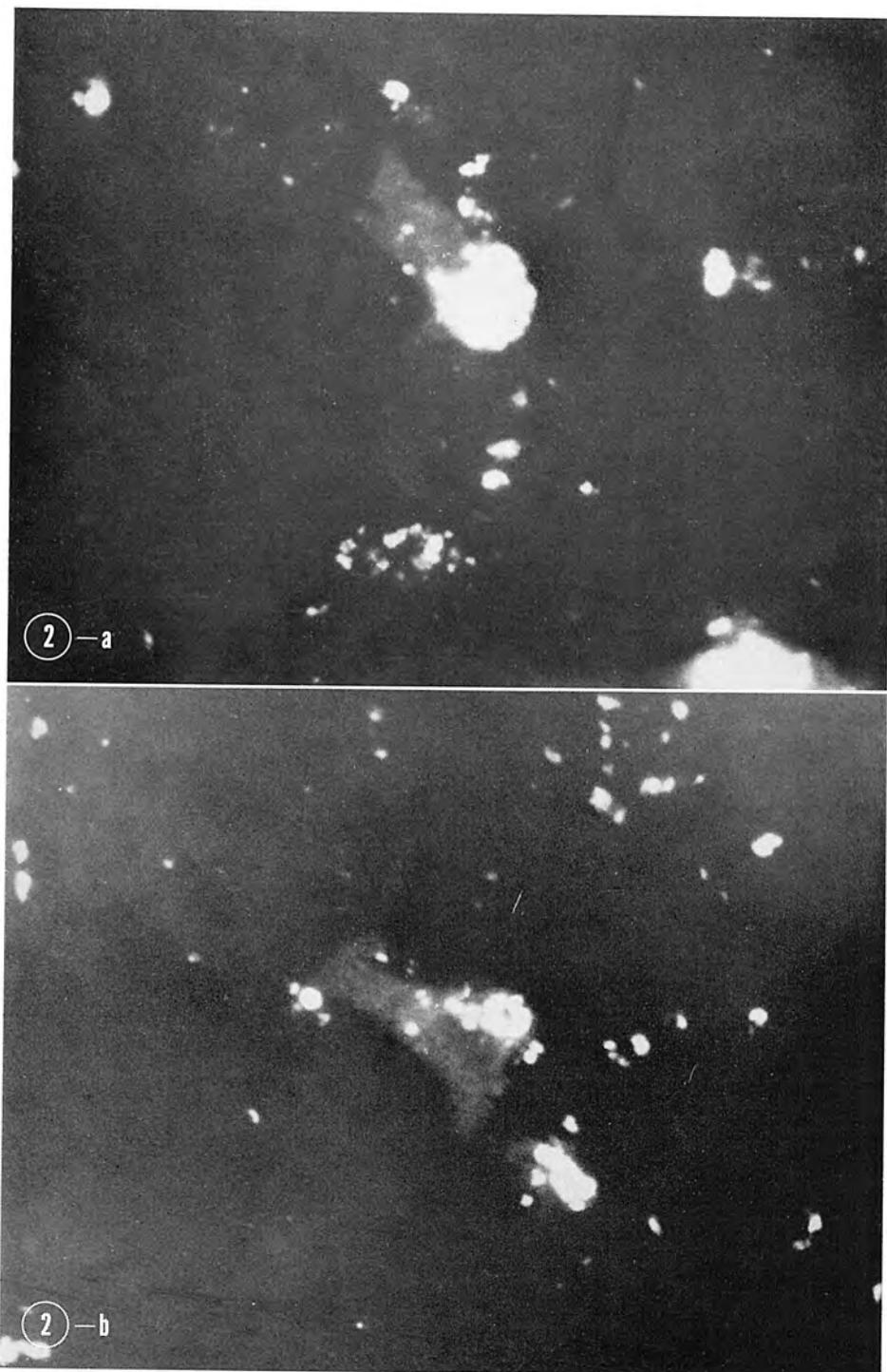
TABLE 1 Criteria for Serodiagnosis of JE

Criterion	Test	Paired specimens		Single specimen
		Rise in titer	Maximum titer	Maximum titer
Certainly positive (++)	CF	4 \times	1: \geq 16	1: \geq 32
	HI	4 \times	1: \geq 320	1: \geq 640
Almost certainly positive (+)	CF	4 \times	1: 8	1: 16
	HI	4 \times	1: 160	1: 320
Probably positive (\pm)	CF	1: < 4	1: 4	1: 8
	HI	4 \times	1: 80	1: 160

Cases that were still negative 13 days after the onset of the disease were designated as "undetermined".

FIGURE 2a Specific Fluorescence in Nerve Cell Stained by the FAT

FIGURE 2b Specific Fluorescence in Nerve Cell Stained by the FAT



any mice within 10 days after inoculation, brain suspensions were made from them and passaged, and then isolated specimens were identified by the FAT.

4. Serologic tests

The HI and complement fixation (CF) tests were conducted by the method of CLARKE and CASALS (1958). NT was carried out in chick embryo culture by the 50 per cent plaque reduction method (PORTERFIELD, 1960: OYA *et al.*, 1967). Serodiagnosis with HI and CF was carried out as described by UCHIYAMA *et al.* (1962) (Table 1).

RESULTS

1. Effectiveness of using the FAT for diagnosis in the epidemiological survey of JE

During 1965-1967, 194 cases whose death was suspected to be due to JE by clinical diagnosis were tested by the FAT, virus isolation and serologic tests in Osaka, and 160 of them were confirmed to have JE. Table 2 shows the number of cases who were JE positive by the three tests, virus isolation and serologic tests or serologic tests alone. The differences of 92 and 52 in the total numbers of cases detected by the different methods indicate the necessity of obtaining brain specimens and application of FAT to them in the epidemiological survey of JE.

2. Detection of JE by the FAT and virus isolation

The relationship between the results by the FAT and those by virus isolation are shown in Table 3. Of 194 cases 127 were confirmed by the FAT and virus isolation. Of 127 cases 122 were detected by the FAT but only 45 cases by virus isolation. Forty of 45 cases which were confirmed by virus isolation were also detected by the FAT. Moreover, 78 negative and 4 undetermined cases by virus isolation were positive by the FAT. This table indicates that the FAT was far more sensitive

TABLE 3 *Detection of JE by the FAT and by Virus Isolation*

		Virus isolation			Total
		Positive	Negative	Undetermined	
FAT	Positive	40	78	4	122
	Negative	4	64	3	71
	Not done	1	0	0	1
Total		45	142	7 ^a	194

^a These cases were undetermined because of accidents and bacterial contamination of brain specimens.

TABLE 2 *Effectiveness of Using the FAT in Diagnosis of JE*

Number of patients tested		1965			1966			1967			Total	
		25		118		51		194				
Number giving positive for JE	FAT+virus isolation+serologic tests (I)		17		106		37		160			
	virus isolation+serologic tests (II)		10		68		30		108			
	serologic tests (III)		4		42		22		68			
Difference in number of detected cases	(I)-(II)		7		38		7		52			
	(I)-(III)		13		64		15		92			

than virus isolation and almost all positive cases could be detected by the FAT alone.

Even when a brain specimen was obtained by puncture 19.5 hours after death, both the FAT and virus isolation demonstrated JE virus antigen in it.

3. Diagnosis by the FAT and by serologic tests

The relationship between results of the FAT and those of serologic tests are shown in Table 4. The agonal stage when the last serum specimens were taken was on the average 4 days after the onset of the disease in the 65 cases which were undetermined serologically but were positive by the FAT. The data show that it is still possible to make diagnosis by the FAT even in cases which die relatively early after the onset of the disease and so are undetermined serologically.

4. Virological tests on autopsy cases in 1966 and 1967

Twenty five autopsy specimens were investigated virologically in 1966 and 1967. The results of the FAT indicate that JE virus antigen was more frequently present in the thalamus than in other brain tissues (Table 5).

5. Practical availability of the brain puncture

The puncture method for obtaining brain specimens in place of autopsy is convenient. To estimate the value of brain punctures, the effectiveness of the two methods in detection

of JE is shown in Table 6. Detection by autopsy is better than by the puncture each year, but in the total over all three years there is no significant difference between detections of JE by the two methods.

6. Clinical course and results of various tests on an extremely protracted case

One case died 151th day after the onset of the disease. The clinical course and results of various tests on this case are shown in Table 7. Three points are of particular interest. I) The FAT demonstrated that JE virus antigen was present in the pons only, while no virus was isolated from the lesion. II) The rise of serum antibody titer was typical for infection with JE and the titer continued at a high level from the 7th to 126th day after the onset of the disease. III) Histopathological examination of brain tissues showed the chronic progressive features of JE (SHIRAKI *et al.*, 1968).

7. Comparison of diagnoses by the three tests during the course of the disease

The detection of JE in the 194 cases who died are shown in Fig. 3 in relation to two factors, namely the three different virological tests and the days after the onset of the disease. Three stages of disease could be differentiated by the FAT. The frequency of positive reactions was low for the first 2 days (Stage I, Positive in 1 of 6 cases), then it was high until the 19th day (Stage II, 115 of 162 cases), and thereafter de-

TABLE 4 Diagnosis by the FAT and by Serologic Tests

		Serologic tests					Total
		Positive	Probably positive	Negative	Unde- termi- ned	Note done	
FAT	Positive	34	7	1	65	15	122
	Negative	33	2	4	24	8	71
	Not done	1	0	0	0	0	1
Total		68	9	5	89	23 ^a	194

^a Specimens were not obtained

TABLE 5 *Virological Tests on Autopsy Cases in 1966 and 1967*

Case	Age	Days after onset	Th	Nig	Pons	Amm	Cort	Others	Sero
6-110	51	2	-/-	-/-		-/-			ND
6-111	72	6	+/+	+/-		+/+			ND
6-112	69	6	+/- ^a	-/-		-/-	+/+		ND
6-113	74	7	+/-	+/-		+/+			ND
6-114	69	3	-/-	-/-		+/+			ND
6-115	44	8	+/-	-/-		+/-	+/-		ND
6-116	84	25	-/-	-/-		-/-			+
6-117	68	23	+/-	+/-		+/-	+/-		ND
6-118	66	151	-/-		+/-	-/-	-/-		+
7- 36	24	7	-/-				-/-		ND
7- 37	56	5	-/-				-/-		ND
7- 38	55	7	+/-	-/-			-/-	-/-	ND
7- 39	75	5					-/-		ND
7- 40	82	1	-/-	-/-				-/-	ND
7- 41	85	10						-/-	ND
7- 42	26	29	-/-	-/-		-/-	-/-		-
7- 43	57	6	-/-	-/-	+/-		+/-		+
7- 44	68	9	+/-	-/-	-/-				ND
7- 45	81	7	+/-	-/-	+/-	-/-			ND
7- 46	33	11	-/-						ND
7- 47	71	5	+/+	+/+	+/+	+/+			ND
7- 48	58	5	+/-	-/-	-/-	+/-			+
7- 49	64	9	+/-	-/-	-/-	-/-			+
7- 50	54	5	-/-	-/-	-/-	-/-			ND
7- 51	52	5	+/-	-/+	-/-	-/-			ND

a +/- FAT was positive and virus isolation was negative on the same specimen.

Th : thalamus, Nig : substantia nigra, Amm : cornu ammonis, Cort : cortex cerebri, Others : other portions of brain tissues, Sero : serologic tests, ND : could not be determined

TABLE 6 *Detection of JE by the FAT on Specimens Obtained at Autopsy and by Puncture*

Year	1965	1966	1967	Total
Autopsy	2/3 (66.7)	7/9 (77.8)	8/16 (50.0)	17/28 (60.7)
Puncture	12/22 (54.5)	81/109 (74.3)	12/34 ^a (35.3)	105/165 (63.6)
Total	14/25 (56.0)	88/118 (74.6)	20/50 (40.0)	122/193 (63.2)

Numerator : Number of positive cases. Denominator : Number of cases tested.

a 1 of 35 cases was not tested because of an accident.

TABLE 7 *Clinical Course and Results of Various Tests on Extremely Protracted Case*

Case No. 6-118, female, 66 years old, Vaccination (?)			
Onset 8-13, 1966			
1) Clinical features and treatments			
Days from onset			
1	fever (42°C), incontinence of urine and feces, coma		
3	admission, nutrition through nasal tube until death		
10-148	slight fever (37-38°C)		
149	fever (40.5°C)		
151	death		
2) Autopsy (10 hrs after death)			
brain atrophy (1190 g including cerebellum)			
hemorrhagic spots in the thalamus, pulmonary congestion			
3) Histopathological examination of brain tissues			
chronic progressive features of JE (SHIRAKI <i>et al.</i> , 1968)			
4) FAT			
positive in pons			
5) Virus isolation			
negative			
6) Serologic tests			
Days from onset	HI	CF	NT (\log_{10} antibody titer)
5	1 : <10	1 : <4	1.8
7	1 : ≥ 2560	1 : <4	3.7
21	1 : ≥ 2560	1 : 16	3.8
28	1 : ≥ 2560	1 : 32	3.7
35-56	1 : ≥ 2560	1 : 64	3.4-3.7
65-70	1 : 1280	1 : 32	3.4-3.1
84	1 : 1280	1 : 16	2.9
91	1 : 1280	1 : 8	2.8
95-112	1 : 640	1 : 8	3.0-2.5
119	1 : 640	1 : 4	3.1
126	1 : 640	1 : 8	Not done

TABLE 8 *Results of Tests for Viremia and NT on JE patients*

Days from onset	1	2	3	4	5	6	7-	Total
No. of the first specimen taken	1	12	18	11	7	6	9	64
Detection of viremia	0/1	0/13	0/20	0/14	0/15	0/12	0/26	0/101
Mean of NT antibody titer	2.70 ^a	2.46	2.65	2.87	3.27	3.58	2.60	

Numerator : Number of specimens from which JE virus was isolated.

Denominator : Number of specimens tested.

^a Figure represents \log_{10} NT antibody titer of the first specimen taken.

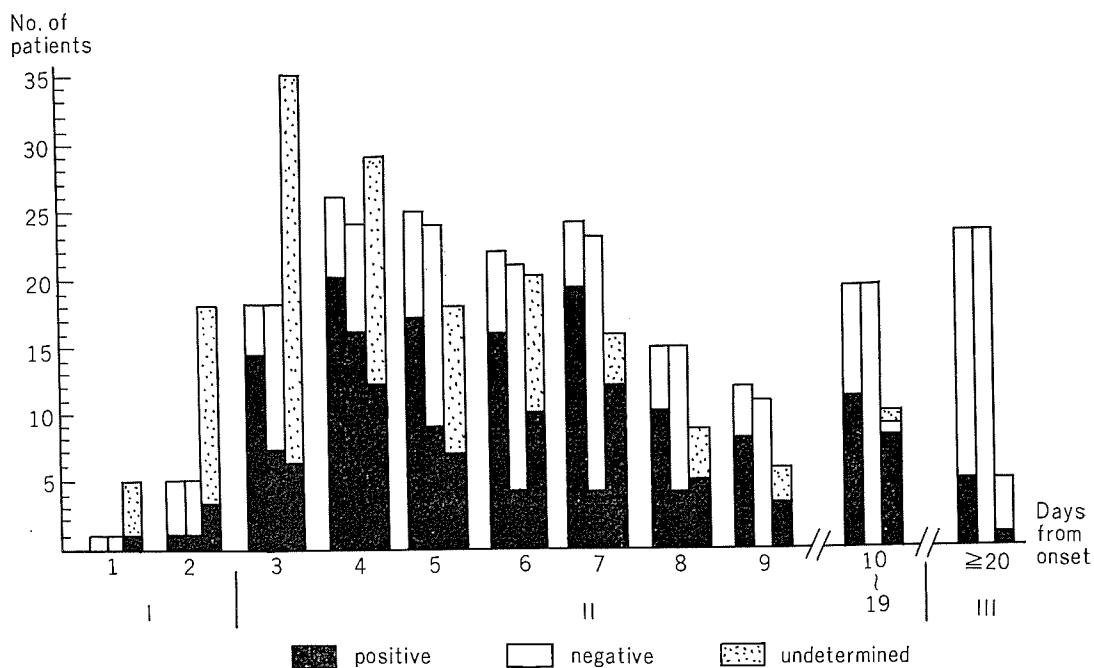


FIGURE 3 Comparison of Diagnoses by the Three Techniques during the Course of the Disease. Shows results of the FAT (left column), virus isolation (central) and serologic tests (right) on 190, 184 and 171 patients, respectively.

Determinations by the FAT and virus isolation were made on specimens obtained after death at various periods after onset of the disease.

In serologic tests, "positive" and "negative" represent patients confirmed on the various days after onset and "undetermined" means that a positive result was still not obtained with the last specimen taken.

creased gradually (Stage III, 5 of 23 cases). Consequently, the FAT seems to be the best method when applied within 28 days, and especially in the early stage of the disease. The virus isolation test was valid for detection of JE within 8 days from the onset while the proportion of positive reactions in serologic tests was less than 50 per cent in the first week of the disease.

8. Results of tests for viremia and NT on JE patients

In 1967, as a preliminary test in detection of viremia by the FAT, viremia in JE patients was studied by the routine virus isolation test but no JE virus could be detected in all 64 patients tested within 10 days after the onset of the disease. This failure may partly have been

due to the high neutralizing antibody titers in the sera (Table 8).

DISCUSSION

The mortality from JE is higher than from other viral infections in Japan and the death usually occurs in the early stage of the disease (TOTANI, 1967). The serological tests were not so effective for diagnosis of these cases and left a high proportion of undetermined cases. These difficulties have been an important problem in the epidemiological survey of JE in Osaka. In the present work, they were overcome to some extent by the combination of two tests, use of an instrument for brain puncture and application of the FAT (Table 2). For laboratory diagnosis of JE the FAT gave a better result

than other virological tests and it was particularly valuable because of its high sensitivity and rapidity. Moreover, it could be used in an early stage of the disease when both virus isolation and serologic tests frequently gave negative results. Diagnosis could frequently be made by the FAT and virus isolation with brain specimens obtained quite a long time after death, suggesting that the immunological reactivity and infectivity of JE virus in brain tissues survives for a considerable time.

Results of various tests on a patient who died 151th day after the onset of the disease (Table 7) were of great interest in three respects, as previously described, and led to various speculations. I) It seems from the FAT and histopathological examination, that JE virus antigen was preserved in the pons area until death, and that reactivation of the virus in remission may cause the chronic progressive features of JE histopathologically (SHIRAKI, 1966). II) From the continued presence of a high antibody titer, the results of the FAT and virus isolation show that non-infectious JE virus particles which have been neutralized by the antibody may be preserved in the pons and react with fluorescent antibody.

Puncture was more convenient than autopsy and could be used for obtaining specimens without injury of the skin of the head. How-

ever, the specimens obtained were very small and their anatomical locations were difficult to identify.

Although the combined use of the FAT and brain puncture greatly improved the diagnostic efficiency, it is still difficult to diagnose cases of JE soon after the onset of the disease. One reason for this is the difficulty in detection of viremia in patients (MITAMURA, *et al.*, 1937: ISHII, 1963). In the present study no viremia could be detected in 64 patients suspected of JE by clinical diagnosis within 10 days after the onset of the disease by the routine virus isolation technique, but in every case the neutralizing antibody titer was high. This does not exclude the possibility of direct detection of viremia by the FAT, because the FAT proved far more sensitive than virus isolation or serologic tests in the present work.

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