Bull. Org. mond. Santé
Bull. Wld Hlth Org. } 1968, **38**, 547-563

Immunotyping of Different Strains of Japanese Encephalitis Virus by Antibody-Absorption, Haemagglutination-Inhibition and Complement-Fixation Tests*

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The immunological characteristics of 26 strains of Japanese encephalitis virus (JEV) isolated in Japan and Malaya between 1935 and 1966 have been investigated mainly by the antibody-absorption variant of the haemagglutination-inhibition test, and to a certain extent also by conventional haemagglutination-inhibition and complement-fixation tests. The antibody-absorption technique shows promise as a routine method for the immunotyping of JEV.

At present, two immunotypes can be distinguished. One comprises 2 strains, Nakayama-NIH and I-58, and is designated as the I-58 immunotype. The other immunotype, JaGAr 01, comprises 17 strains which share the characteristics of the JaGAr 01 strain, including one subline of the Nakayama strain, Nakayama-Yakken. The Nakayama-RFVL strain was found to have the characteristics of both immunotypes. The I-58 immunotype differs more markedly from related arboviruses, such as the Murray Valley encephalitis virus and the West Nile Eg101 strain, than does the JaGAr 01 immunotype.

Evidence is presented which suggests that a given JEV strain can change immunotype on repeated passage through mice.

The immunological typing of Japanese encephalitis viruses (JEV) remains an intractable problem. In their pioneer research on Japanese encephalitis, Mitamura et al. (1936) carried out comparative neutralization tests, but were unable to find significant differences between the strains. Hale & Lee (1954) were the first to demonstrate immunological variation in JEV. Further investigations in the authors' institute showed immunological differences between the Nakayama-NIH strain—recommended by the Ministry of Health and Welfare, Japan, for the manufacture of human vaccine—and a relatively new strain, G-1-Late; these differences were demonstrated by means of the intracerebral protection test (Ogata, 1959) and by complement-fixation (CF), haemagglutination-inhibition (HI), neutralization and intracerebral protection tests (Kobayashi, 1959). Another subline of the Nakayama strain, Nakayama-Yakken, was recommended by the Ministry of Agriculture and Forestry, Japan, for use in a veterinary vaccine; the immunological differences between the two sublines of the Nakayama strain were investigated by Murakami (1961) and Fujie et al. (1962); their findings formed the basis of national assays of the vaccines. Apart from vaccine assays, an inquiry into the existing immunotypes of JEV was considered useful from the diagnostic, ecological and epidemiological points of view. Accordingly, attempts were made to compare systematically some 30 JEV strains, isolated over a span of 30 years, by a combination of *in vitro* immunological techniques. An attempt was also made to determine the immunological relationship of JEV with closely related but distinct arboviruses of group B, without complicating the picture further by stressing minor immunological differences. The results of these investigations are given in the present paper.

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MATERIALS AND METHODS

Viruses

Altogether, 26 strains of JEV and a number of related viruses were studied. There were 22 Japanese and 4 Malayan strains of JEV, listed in Table 1. The 4 Malayan strains were supplied by the Department of Bacteriology, University of Singapore; of these, strain S-705/64, isolated in 1964 in Singapore by Chan & Loh (1966), had been sent to us for confirmative identification. Strains H-58 and I-58 of JEV were isolated by Ohtahara et al. (1959), and later supplied by Okayama University. The other strains of JEV have been maintained in this institute. The Murray Valley encephalitis (MVE) virus, strain name unknown, had been given to this department by the 406 Medical General Laboratory, Tokyo, in the 1950s. The West Nile (WN) strain, Eg101, was given by the Rockefeller Foundation Virus Laboratories (RFVL), New York, through Dr Jordi Casals, together with their prototype JEV strain, Nakayama-RFVL. A St Louis encephalitis (SLE) strain was also studied.

TABLE 1

JAPANESE ENCEPHALITIS VIRUS STRAINS STUDIED

No.	Strain designation	Isolation			Test result			Immunotype
		Year	Place	Source	HI	CF	Antibody absorption	
1	Nakayama-NIH	1935	Tokyo	Human CSF [a]	+	+	+	I-58
2	Nakayama-RFVL	1935	Tokyo	Human CSF [a]	+	+	+	Intermediate
3	Nakayama-Yakken	1935	Tokyo	Human CSF [a]	+	+	+	JaGAr 01
4	Kalinina	1935	Tokyo	Human brain			+	JaGAr 01
5	G-1-Early	1949	Tokyo	Human brain	+		+	JaGAr 01
6	G-1-Late	1949	Tokyo	Human brain	+		+	JaGAr 01
7	Muar	1952	Malaya	Human brain	+		+	(JaGAr 01)
8	Tengah	1952	Singapore	Human brain	+	+	+	(JaGAr 01)
9	GH3/52	1952	Singapore	Human brain	+		+	JaGAr 01
10	Mochizuki	1953	Okayama	Human brain	+		+	JaGAr 01
11	Igaya	1955	Tokyo	Human brain			+	JaGAr 01
12	Ikeda	1955	Tokyo	Human brain	+			
13	Otsubo	1956	Tokyo	Human brain	+			
14	Tanaka	1957	Tokyo	Human brain			+	JaGAr 01
15	TOP	1957	Tokyo	Human brain	+			
16	Hotta	1958	Tokyo	Human brain			+	JaGAr 01
17	Yachida	1958	Tokyo	Human brain			+	JaGAr 01
18	I-58	1958	Okayama	Human brain	+	+	+	I-58
19	H-58	1958	Okayama	Human brain	+		+	JaGAr 01
20	JaGAr 01	1959	Gunma	*Culex tritaeniorhynchus*	+	+	+	JaGAr 01
21	JaGAr 21	1959	Gunma	*Culex tritaeniorhynchus*			+	JaGAr 01
22	Kitano	1959	Osaka	Human brain	+			
23	JaGAr 15460	1960	Gunma	*Culex tritaeniorhynchus*	+		+	JaGAr 01
24	Konishi	1960	Kyoto	Human brain			+	JaGAr 01
25	S-705/64	1964	Singapore	Human blood		+	+	JaGAr 01
26	JaTH 166	1966	Tokyo	Human brain			+	JaGAr 01

[a] CSF = cerebrospinal fluid.

Antigen for HI and CF tests

The antigens were prepared from infected suckling mouse brains by acetone-ether or sucrose-acetone extraction (Clarke & Casals, 1958); the antigen was usually lyophilized and stored in ampoules at −20°C.

Preparation of antiserum

Guinea-pigs, or more frequently mice, were used for immunization. The method of vaccination varied according to the pathogenicity of the virus strain concerned. In the case of WN, Russian spring-summer encephalitis (RSSE), Powassan and Negishi viruses, the mice were vaccinated once intraperitoneally with β-propiolactone-inactivated vaccine; otherwise, animals were vaccinated repeatedly with live virus. The animals were bled partially a week following 1–2 vaccinations, or bled to death a week after 3–5 vaccinations. The former procedure gave " primary " serum, and the latter " hyperimmune " serum. The sera were dispensed into short tubes and stored at −20°C until use.

HI test

The conventional procedure for HI tests (Clarke & Casals, 1958) was followed, with slight modifications which have already been described in previous reports (Okuno et al., 1961). Sera were treated with acetone, absorbed with goose cells to remove spontaneous agglutinin against the cells, and stored in rubber-stoppered tubes at 4°C until use. It was confirmed experimentally that the HI titres of such sera remained unchanged during a year of storage.

For each test, serial twofold dilutions of serum were first prepared in master tubes, starting from a concentration of 1 : 20, and then distributed in 0.2-ml amounts into the plate wells; 16 haemagglutination units of antigen in 0.2 ml were added to each well. As far as a given batch of the treated serum was concerned, titration errors remained within twofold.

In the case of serum derived from antibody absorption, the absorbed 1 : 10 serum was treated with 12.5% kaolin instead of acetone; apart from this, the test was carried out in the usual manner.

CF test

CF tests were performed two-dimensionally with serial dilutions of serum and antigen in the presence of 1.7–2.2 units of complement. The Disposo CF tray (Ace Scientific Co., New Jersey, USA), with accompanying dropping-syringe and needles, was employed. Sera were inactivated at 56°C for 30 minutes before testing. The CF titre was taken as the highest dilution of serum or antigen which gave 25% haemolysis or less.

The antibody-absorption test

The original technique of Clarke (1960) was followed with very little modification. All sera had been prepared by the repeated immunization of 7-week-old mice. Absorbing viruses were obtained from 20% infected suckling mouse brain suspension by centrifugation at 11 000 rev/min, followed by further centrifugation of the resulting supernatant at 40 000 rev/min for 2 hours (using 11.5-ml portions in Lusteroid tubes in the Spinco L rotor No. 40). For the actual test, a viral pellet prepared as described above was first homogenized with 3 ml of 1 : 10 serum employing a Teflon plunger (Arthur H. Thomas Co., Philadelphia, Pa., USA) and held overnight at 4°C; the remaining viruses were then removed by centrifugation at 40 000 rev/min; the resulting absorbed serum (1 : 10) was treated as described above for the HI test, and its HI titre against various antigens determined. When the homologous HI titre of a given serum fell below 1 : 20, this was interpreted as indicating at least unilateral correspondence between the serum antibody and the virus. When absorption of antibody with a single pellet failed to reduce the homologous HI titre below 1 : 20, double or quadruple amounts of virus were used. If the HI titre was 1 : 20 or higher, even after absorption with 4 pellets, the serum antibody and the virus were considered not to be identical.

The amount of specific antibody remaining was expressed by the reduction in the HI titre of a given serum after absorption with the smallest amount of brain virus needed for the complete absorption of antibody homologous to the virus. Usually, a single pellet was found to be sufficient for 3 ml of 1 : 10 serum; this corresponds to approximately 7.6 g of brain per ml of undiluted serum.

RESULTS

Typing of JEV strains by the HI test employing primary and hyperimmune sera against group-B arboviruses

The results of HI tests on 17 JEV strains and 3 other strains are given in Fig. 1 and Fig. 2. It may be seen from Fig. 1 that the antigens of 4 strains—namely, Nakayama-NIH, Nakayama-RFVL, Nakayama-Yakken and JaGAr 15460—were inhibited relatively poorly by most sera. The I-58 strain shows

a similar tendency in Fig. 2. In other words, those 5 strains are less reactive than the others. In contrast to this, strains like Mochizuki or JaGAr 01 reveal HI titres which are generally 2 or 3 dilutions higher than those of such strains as Nakayama-NIH or I-58.

Primary serum No. 1 (anti-Nakayama-NIH) was found to be extremely specific, giving a titre of from 1 : 20 to 1 : 80 with only 2 antigens, the homologous Nakayama-NIH and I-58. On the other hand, all strains gave titres with the JaGAr 01 primary serum, although the titre against Nakayama-NIH and Nakayama-RFVL antigens was only 1 : 20—4-fold lower than the homologous titre. It would thus seem to be justified to separate 2 strains, Nakayama-NIH and I-58, from all the others as representing one immunotype. However, further immunological typing of the strains is not easy because of heavy cross-inhibition among group-B arboviruses in HI tests. As may be seen from Fig. 2, it is even difficult to distinguish between JEV and non-JE viruses such as MVE, WN and SLE by this type of HI test.

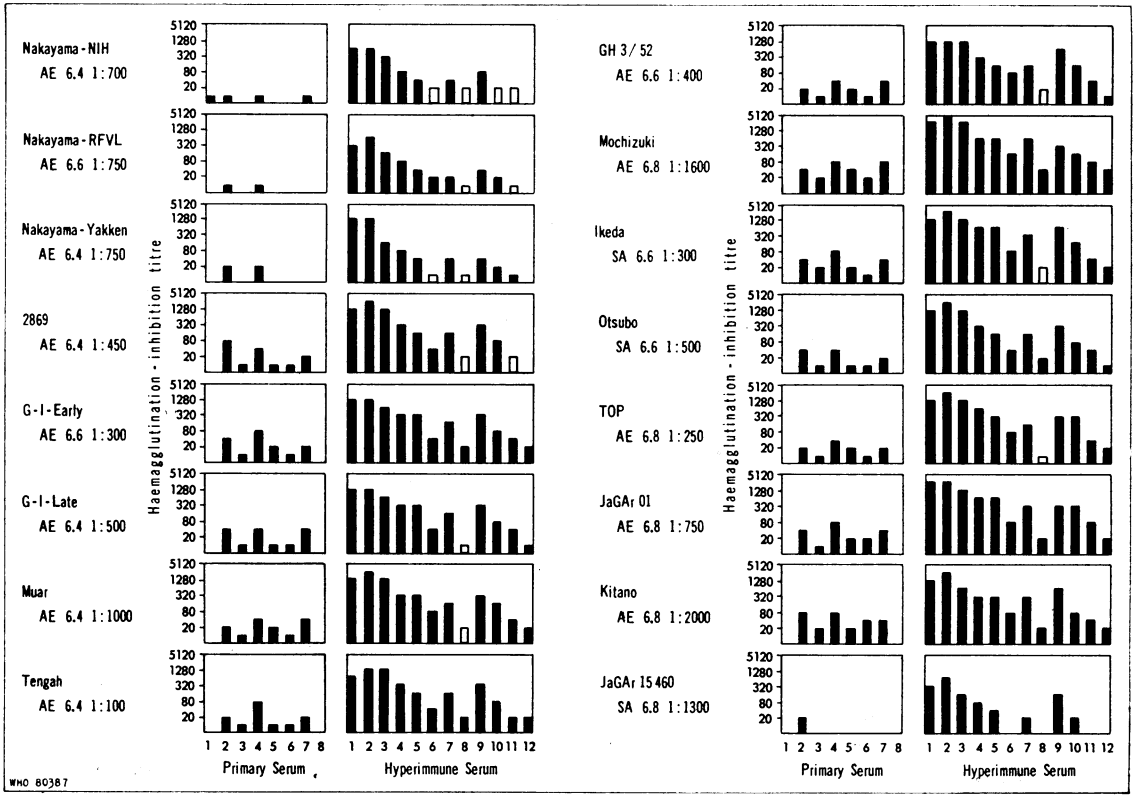
Eleven additional antigens of JEV strains were also tested against a limited number of the same sera; the results for these are not included in Fig. 1 and Fig. 2. Among those, strain H-58 closely resembled I-58 in its poor reactivity, while the other 10 appeared to be more reactive.

Cross-HI tests of the hyperimmune sera and antigens against Nakayama-NIH and JaGAr 01 strains

It was clear from the results described above that the antigens of some strains gave uniformly low titres

FIG. 1

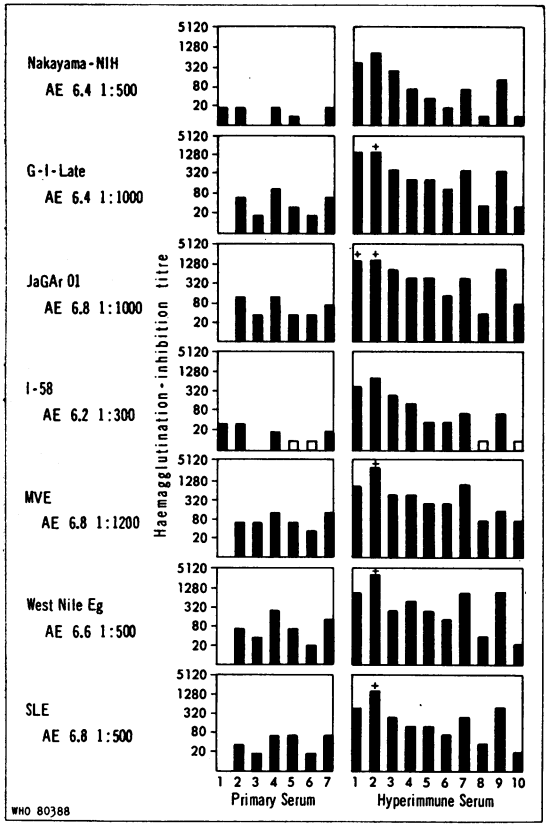
HI PROFILES OF 16 STRAINS OF JAPANESE ENCEPHALITIS VIRUS[a]



[a] Open columns indicate that the HI titre found was sometimes less than the value given. The following details are given for each antigen: extraction method (AE=acetone-ether extraction, SA=sucrose-acetone extraction); optimum pH; and test dilution. The significance of the numbers used to designate antisera is as follows. *Primary sera:* 1=Nakayama-NIH; 2=JaGAr 01; 3= MVE; 4=WN Eg 101; 5=SLE; 6=Negishi; 7=Modoc; 8=Dengue 1. *Hyperimmune sera:* 1=JaGAr 01; 2=G-1-Late; 3=MVE; 4=WN Eg 101; 5=SLE; 6=RSSE; 7=Negishi; 8=Powassan; 9=Modoc; 10=Dengue 1; 11=Dengue 2; 12=YF 17D.

FIG. 2

HI PROFILES OF 4 STRAINS OF JAPANESE ENCEPHALITIS VIRUS AND OF 3 RELATED ARBOVIRUSES [a]



[a] A cross (+) above a column indicates that the HI titre found was sometimes higher than that given. For the meaning of other signs and symbols, see footnote to Fig. 1. The numbers designating antisera are the same in both figures, except that Hyperimmune Serum 10 is YF 17D.

against most of the sera, suggesting a possible reactivity variation like the P–Q variation of influenza A virus which was discovered by van der Veen & Mulder (1950). The results of repeated cross-HI tests between representative reactive and less-reactive strains are summarized in Table 2.

Ten independent tests were carried out, using 3 batches of each antiserum; the results are highly reproducible. The Nakayama-NIH antisera gave nearly the same titre with both antigens. On the other hand, a clear-cut difference was observed between the homologous and heterologous titres of JaGAr 01 antisera against both antigens. This would

TABLE 2

HOMOLOGOUS AND HETEROLOGOUS HI TITRES OF "REACTIVE" (JaGAr 01) AND "LESS REACTIVE" (NAKAYAMA-NIH) JEV STRAINS

Test No.	Serum	Lot No.	HI titre against 8 units of antigen	
			Nakayama-NIH	JaGAr 01
5	Nakayama-NIH	1	1 280	1 280
7			2 560	2 560
8			2 560	2 560
10			1 280	1 280
12			2 560	1 280
16		2	5 120	2 560
170		3	1 280	640
2	JaGAr 01	1	1 920	15 360
2			1 280	20 480
7			640	5 120
7			1 280	5 120
10			640	2 560
16			640	2 560
170		2	1 280	⩾5 120
18			1 280	5 120
113		3	480	3 840

seem to suggest that the relationship between the JaGAr 01 and Nakayama-NIH strains resembles that between the P and Q phases of influenza A, respectively.

Cross-CF tests of JEV and other group-B arboviruses

In earlier studies (Kobayashi, 1958; Okuno et al., 1961), CF tests employed on a limited scale indicated no notable strain differences among JEV. It was also reported (Kobayashi et al., personal communication, 1960) that the majority of Japanese encephalitis patients yielded approximately the same CF antibody level against both Nakayama-NIH and JaGAr 01 antigens. Since the micro-CF technique with Disposo trays enables large numbers of titrations to be carried out, the authors set up a two-dimensional CF test to check the results obtained previously.

Ten virus strains were tested in 4 titration series with 9 control titrations. The range of technical error was usually within a twofold dilution. Difficulties have been known to arise in comparison of fixation profiles when the potencies of antigen and antiserum are variable. The fixation profiles found here were

therefore adjusted to correspond to homologous systems uniformly composed of 8 units of antigen and antiserum. The adjusted CF profiles are shown in Fig. 3.

The discrete nature of SLE can easily be recognized, as already stated by earlier investigators. WN was closer to JEV than SLE, and MVE was even closer to JEV. All the JEV strains yielded more or less similar CF patterns. However, closer inspection of Fig. 3 shows that 4 strains of JEV—namely, Nakayama-RFVL, Nakayama-Yakken, S-705/64 and JaGAr 01—appear to resemble one another particularly closely, though the JaGAr 01 antiserum is extremely reactive, even after adjustment. On the other hand, sera against the I-58 and Nakayama-NIH strains discriminated between JaGAr 01 antigen and the homologous antigen. In fact, the only clear separation between I-58 and Nakayama-NIH (sera) is given by MVE antigen. The CF results thus also suggested the existence of at least 2 immunological types of JEV. As to the Tengah strain, although most cross-CF tests were not completed, the serum separated the homologous and Nakayama-RFVL antigens to an appreciable extent; this is in agreement with the report of Hale & Lee (1954).

The antibody-absorption test for antigenic analysis of JEV

General. The preceding results indicated that a few JEV strains were distinct from the rest. However, as long as these strains were examined in conventional HI tests, the variability in the HI reactivity of each JEV strain was superimposed upon genuine immunological variations. In the CF tests, direct comparison of fixation profiles was often impossible because of fluctuation of the potencies of antigen and antiserum. The antibody-absorption modification of the HI test, developed by Clarke (1960), was considered to be more suitable because of its simplicity, better reproducibility and clear-cut results.

Relationship between Nakayama-NIH and JaGAr 01 strains. The results of antibody-absorption tests carried out with these two strains are summarized in Table 3. As described above, in the conventional HI test Nakayama-NIH serum gave nearly an identical titre (1 : 1280) with each antigen employed

FIG. 3

CROSS-CF PROFILES OF 7 STRAINS OF JAPANESE ENCEPHALITIS VIRUS AND 3 RELATED ARBOVIRUSES

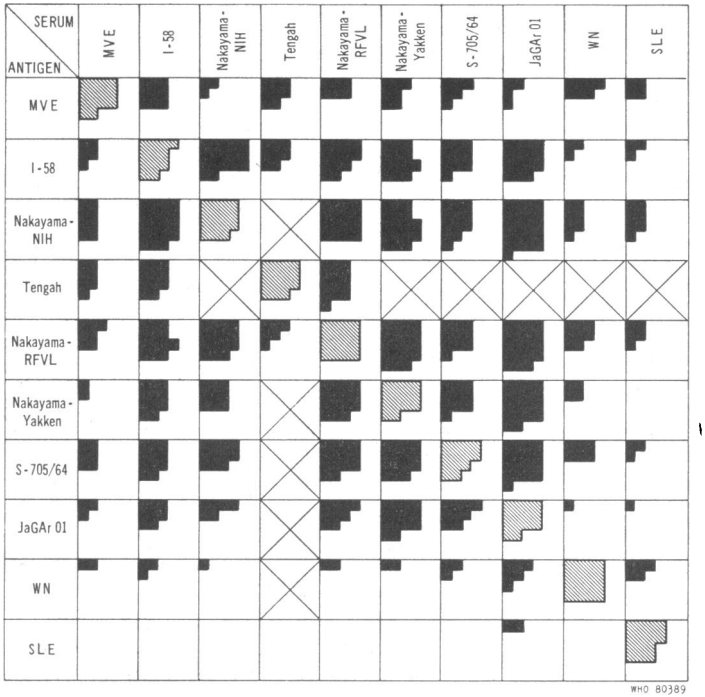


TABLE 3

HOMOLOGOUS AND HETEROLOGOUS ANTIBODY-ABSORPTION HI TITRES OF NAKAYAMA-NIH AND JaGAr 01 STRAINS [a]

Test No.	Serum		Absorption		HI titre against 8 units of antigen	
	Against strain	No. of injections [b]	Virus	No. of pellets [c]	Nakayama-NIH	JaGAr 01
5	Nakayama-NIH	6	None	–	**1 280**	**1 280**
			Nakayama-NIH	1	**<20**	**<20**
			JaGAr 01	1	**40**	**<20**
				2	**40**	**<20**
				4	**40**	**<20**
2	JaGAr 01	6	None	–	**1 920**	**15 360**
			JaGAr 01	1	**<15**	**<15**
			Nakayama-NIH	1	**<15**	**30**
				2	**<15**	**30**
				4	**<15**	**30**
113	JaGAr 01 [d]	5	None	–	**480**	**3 840**
			Nakayama-NIH	1	**<15**	**60**
				2	**<15**	**60**
				4	**<15**	**60**

[a] Titres in bold type indicate the amount of residual specific antibody.

[b] The number of injections per mouse used in the production of the serum.

[c] Each virus pellet corresponded to 6.6 g–7.6 g of infected suckling mouse brain. The chosen number of pellets was used to absorb 3 ml of 1 : 10 serum.

[d] Plaque-purified.

while JaGAr 01 serum separated homologous and heterologous antigens remarkably clearly. With either antigen, homologous absorption with 1 viral pellet as described above removed all antibody. In fact, this was usually true with the 9 JEV strains tested thus far; difficulties with the absorption of homologous antibody as encountered by Clarke (1960) in the case of yellow fever virus were not encountered. In spite of a twofold or fourfold increase in the amount of absorbing virus, the specific antibody for each strain remained unabsorbed by heterologous virus. With the minimum amount of heterologous absorbing virus (1 pellet), Nakayama-NIH serum retained 3.1% of specific antibody, and JaGAr 01 retained 0.2% to 1.5%.

I-58 strain. This strain has low HI reactivity, and resembled Nakayama-NIH in both HI and CF tests, as described above. The I-58 antiserum which was prepared by 6 live-virus vaccinations had a rather low HI titre (1 : 640). Absorption of the serum with JaGAr 01 brain virus reduced the homologous as well as the heterologous antibody level until no antibody was left following absorption with 4 pellets of the brain virus (Table 4). On the other hand, I-58 virus failed to absorb JaGAr 01 specific antibody even with 4 pellets, whereas a single pellet of the virus could remove all the antibodies of Nakayama-NIH serum. As a whole, these results indicate almost complete identity of the I-58 strain with the Nakayama-NIH strain.

Kalinina strain. This strain of JEV was isolated from a Russian patient in Tokyo in 1935, the year when the well-known Nakayama strain was also isolated. In other words, Kalinina is one of the oldest JEV strains available. This strain has undergone at least 61 adult mouse brain passages. Table 5 summarizes the results of 2 individual experiments with this strain. The Kalinina strain was found to be distinct from the Nakayama-NIH: an appreciable residue of specific antibody was left when Nakayama-NIH serum was absorbed with an adequate amount of Kalinina virus. On the other hand, the Kalinina specific antibody level was only 1 : 10 when the serum was absorbed with 4 pellets of Nakayama-NIH virus.

TABLE 4. IMMUNOLOGICAL TYPING OF I-58 STRAIN BY ANTIBODY-ABSORPTION TEST [a]

Test No.	Serum		Absorption		HI titre against 8 units of antigen		
	Against strain	No. of injections [b]	Virus	No. of pellets [c]	Nakayama-NIH	I-58	JaGAr 01
7	I-58	6	None	–	640	640	640
			I-58	1	<20	<20	<20
			Nakayama-NIH	1	<20	<20	<20
			JaGAr 01	1	20	20	20
				2	40	20	<20
				4	<20	<20	<20
7	Nakayama-NIH	6	None	–	2 560	1 280	2 560
			I-58	1	<20	<20	<20
7	JaGAr 01	6	None	–	640	320	5 120
			I-58	1	<20	<20	**80**
				2	<20	<20	**20**
				4	<20	<20	**20**

[a] Titres in bold type indicate the amount of residual specific antibody.

[b] The number of injections per mouse used in the production of the serum.

[c] Each virus pellet corresponded to 6.6 g–7.6 g of infected suckling mouse brain. The chosen number of pellets was used to absorb 3 ml of 1 : 10 serum.

TABLE 5. IMMUNOLOGICAL TYPING OF KALININA STRAIN BY ANTIBODY-ABSORPTION TEST [a]

Test No.	Serum		Absorption		HI titre against 8 units of antigen				
	Against strain	No. of injections [b]	Virus	No. of pellets [c]	Nakayama-NIH	Nakayama-RFVL	Nakayama-Yakken	JaGAr 01	Kalinina
16	Kalinina	5	None	–	320	320	640	1 280	640
			Kalinina	1	<20	<20	<20	<20	<20
			Nakayama-NIH	1	<20	<20	<20	**20**	<20
				2	<20	<20	<20	**20**	<20
				4	<20	<20	<20	**40**	<20 [d]
			JaGAr 01	1	<20	<20	<20	<20	<20
10	Nakayama-NIH	6	None	–	1 280			1 280	320
			Kalinina	1	**80**			<20	<20
				2	**80**			<20	<20
16	Nakayama-NIH	5	None	–	5 120	2 560	2 560	2 560	1 280
			Kalinina	4	**80**	<20	<20	<20	<20
10	JaGAr 01	6	None	–	640			2 560	640
			Kalinina	2	<20			<20	<20

[a] Titres in bold type indicate the amount of residual specific antibody.

[b] The number of injections per mouse used in the production of the serum.

[c] Each virus pellet corresponded to 6.6 g–7.6 g of infected suckling mouse brain. The chosen number of pellets was used to absorb 3 ml of 1 : 10 serum.

[d] In fact the residual titre was 1 : 10.

The fact that a higher antibody titre was detected against JaGAr 01 antigen may be explained by a difference in reactivity between JaGAr 01 and Kalinina antigens; in any case, this would seem to confirm the close similarity of the Kalinina and JaGAr 01 strains.

JaGAr 15460 strain. In the conventional HI tests, the antigen of this strain was found to be extremely poor in reacting with group-B sera, like the antigens of Nakayama-NIH and I-58. However, the Nakayama-NIH specific serum failed to inhibit the antigen of this strain, whereas JaGAr 15460 serum could inhibit the homologous and I-58 antigens. It was hoped that this apparent discrepancy would be explained by the antibody-absorption tests.

As can be seen from Table 6, the relation between the JaGAr 15460 and Nakayama-NIH strains is not completely reciprocal. The antibody against JaGAr 15460 was completely absorbed by either Nakayama-NIH or JaGAr 01. However, Nakayama-NIH antiserum with a titre of 1 : 2560 still had a specific antibody titre of 1 : 80 after the serum had been absorbed with JaGAr 15460 virus. Since JaGAr 01 antiserum retained no specific antibody after absorption with JaGAr 15460 virus, it may be concluded that JaGAr 15460 is more closely related to JaGAr 01 than to Nakayama-NIH or I-58.

G-1-Early and G-1-Late strains. The G-1 strain consists of 2 sublines—namely,G-1-Early which has undergone 7 adult and 3 suckling mouse brain passages, and G-1-Late with 189 adult and 1 suckling mouse brain passages. Comparison of these sublines against other group-B sera in conventional HI tests, as described above, gave the impression that the 2 sublines were identical. Nevertheless, antibody-absorption tests were performed to confirm this finding, since some characteristics of the G-1 sublines seemed to have changed during the large number of adult mouse brain passages which they had undergone (Oya, 1963; Okuno et al., 1965).

The results given in Table 7 indicate the identity of the G-1 and JaGAr 01 strains and the relative stability of the immunological properties of the former. However, it should be noted that there were slight differences between the Early and Late sublines when their antisera were absorbed with increasing amounts of Nakayama-NIH virus. The G-1-Early serum retained a specific antibody titre of 1 : 80; on the other hand, the Late serum eventually left no antibody although the specific antibody titre was still quite high after absorption with 1 to 2 pellets of the brain virus. It should also be mentioned that the G-1-Early serum absorbed with Nakayama-NIH virus gave a homologous antigen titre of 1 : 80, as compared with a titre of 1 : 10 against G-1-Late antigen. In brief, G-1-Late appears to be closer to Nakayama-NIH than to G-1-Early, although both

TABLE 6. IMMUNOLOGICAL TYPING OF JaGAr 15460 STRAIN BY ANTIBODY ABSORPTION-TEST [a]

Test No.	Serum		Absorption		HI titre against 8 units of antigen		
	Against strain	No. of injections [b]	Virus	No. of pellets [c]	Nakayama-NIH	JaGAr 15460	JaGAr 01
9	JaGAr 15460	6	None	—	640	1 280	2 560
			JaGAr 15460	1	<20	<20	<20
			Nakayama-NIH	1	<20	<20	<20
			JaGAr 01	1	<20	<20	<20
8	Nakayama-NIH	6	None	—	2 560	2 560	2 560
			JaGAr 15460	1	**80**	<20	<20
				2	**40**	<20	<20
				4	**40**	<20	<20
7	JaGAr 01	6	None	—	1 280	2 560	5 120
			JaGAr 15460	1	<20	<20	<20

[a] Titres in bold type indicate the amount of residual specific antibody.

[b] The number of injections per mouse used in the production of the serum.

[c] Each virus pellet corresponded to 6.6 g–7.6 g of infected suckling mouse brain. The chosen number of pellets was used to absorb 3 ml of 1 : 10 serum.

TABLE 7

IMMUNOLOGICAL TYPING OF G-1-EARLY AND G-1-LATE STRAINS BY ANTIBODY-ABSORPTION TEST

Test No.	Serum		Absorption		HI titre against 8 units of antigen				
	Against strain	No. of injections [a]	Virus	No. of pellets [b]	Nakayama-NIH	I-58	G-1-Early	G-1-Late	JaGAr 01
17	Nakayama-NIH	6	None	–	2 560	1 280	1 280	640	1 280
			G-1-Early	1	640	320	<10		20
			G-1-Early	2	160	40	<10		10
			G-1-Early	4	160	40	<10		<10
			G-1-Late	1	≥640	≥640		40	80
			G-1-Late	2	320	80		<10	10
			G-1-Late	4	≥640	160		<10	20
17	JaGAr 01	6	None	–	1 280	320	2 560	2 560	2 560
			G-1-Early	1			<10	<10	20
			G-1-Late	1			<10	<10	<10
17	G-1-Early	4	None	–	1 280	320	5 120	2 560	2 560
			Nakayama-NIH	1	<10		80	10	20
			Nakayama-NIH	2	<10		80	10	20
			Nakayama-NIH	4	<10		80	40	80
9	G-1-Early	4	None	–			2 560		2 560
			JaGAr 01	1			<10		<10
17	G-1-Late	8	None	–	1 280	230	2 560	2 560	2 560
			Nakayama-NIH	1	<10		80	80	160
			Nakayama-NIH	2	<10		80	80	160
			Nakayama-NIH	4	<10		<10	<10	<10
9	G-1-Late	8	None	–				2 560	2 560
			JaGAr 01	1				<10	<10

[a] The number of injections per mouse used in the production of the serum.

[b] Each virus pellet corresponded to 6.6 g–7.6 g of infected suckling mouse brain. The chosen number of pellets was used to absorb 3 ml of 1 : 10 serum.

G-1 strains obviously belong to another immunotype, represented by JaGAr 01.

Nakayama-NIH, Nakayama-RFVL and Nakayama-Yakken. The relationship between the Nakayama sublines is more complicated than that between the G-1 sublines. Three sublines are available at the moment. Nakayama-NIH, which has been maintained at the National Institute of Health, Tokyo, through more than a hundred adult mouse brain passages, has been used exclusively for production of the inactivated human vaccine in Japan and China (Taiwan). Shortly after 1945, the strain was supplied to the National Veterinary Assay Laboratory (Yakken) of the Ministry of Agriculture and Forestry. It was passed mainly in adult mouse brain for the production of veterinary vaccine and for the potency assay of this vaccine by a modification of Sabin's method (Sabin et al., 1943). The other strain, supplied by the Rockefeller Foundation Virus Laboratories (RFVL) to the authors' laboratory in 1958, is believed to have undergone the smallest number of adult mouse brain passages. Differences in biological properties between these sublines have been reported elsewhere (Oya, 1963; Okuno et al., 1965); however,

it was expected that antibody-absorption tests would largely, if not entirely, indicate the immunological identity of the 3 sublines. The results, shown in Table 8, were quite surprising.

When Nakayama-NIH serum was absorbed with varying amounts of Nakayama-Yakken virus, an appreciable titre (1 : 160) of antibody specific to Nakayama-NIH remained unabsorbed. On the other hand, absorption of Nakayama-Yakken serum with Nakayama-NIH virus left unabsorbed antibody only when 1 pellet of absorbing virus was used, but no longer when 2 pellets were used. Thus, it became clear that the Nakayama-NIH and Nakayama-Yakken strains were immunologically distinct. It was shown by a later antibody-absorption test that the Nakayama-Yakken strain was substantially identical with the JaGAr 01 strain.

The relationship of the Nakayama-RFVL strain with the Nakayama-NIH and Nakayama-Yakken strains was also investigated. When Nakayama-RFVL was employed as absorbing virus, antibody was completely removed from both Nakayama-NIH and Nakayama-Yakken serum, while both Nakayama-NIH and Nakayama-Yakken virus eliminated all antibody from Nakayama-RFVL serum. This apparent identity of Nakayama-RFVL with both of the two other distinct sublines might be explained on the assumption that Nakayama-RFVL is of an immunotype intermediate between Nakayama-NIH and Nakayama-Yakken.

S-705/64 strain from Singapore. Table 9 demonstrates that this Singapore strain, isolated from the blood of a febrile child, is immunologically closer to JaGAr 01 than to I-58.

TABLE 8

IMMUNOLOGICAL COMPARISON OF THE 3 SUBLINES OF THE NAKAYAMA STRAIN BY ANTIBODY-ABSORPTION TEST

Test No.	Serum		Absorption		HI titre against 8 units of antigen		
	Against strain	No. of injections [a]	Virus	No. of pellets [b]	Nakayama-NIH	Nakayama-RFVL	Nakayama-Yakken
13	Nakayama-NIH	6	None	–	2 560	2 560	2 560
			Nakayama-NIH	1	<20	<20	<20
			Nakayama-RFVL	1	<20	<20	<20
			Nakayama-Yakken	1	160	<20	<20
				2	80	<20	<20
14	Nakayama-NIH	5	None	–	5 120	5 120	2 560
			Nakayama-Yakken	1	160	<20	<20
				2	160	<20	<20
				4	160	<20	<20
13	Nakayama-RFVL	6	None	–	640	1 280	640
			Nakayama-RFVL	1	<20	<20	<20
			Nakayama-NIH	1	<20	<20	<20
			Nakayama-Yakken	1	<20	<20	<20
15	Nakayama-Yakken	5	None	–	320	640	1 280
			Nakayama-Yakken	1	<20	<20	<20
			Nakayama-NIH	1	<20	<20	40
				2	<20	<20	<20
			Nakayama-RFVL	1	<20	<20	<20

[a] The number of injections per mouse used in the production of the serum.

[b] Each virus pellet corresponded to 6.6 g–7.6 g of infected suckling mouse brain. The chosen number of pellets was used to absorb 3 ml of 1 : 10 serum.

TABLE 9

IMMUNOLOGICAL TYPING OF S-705/64 STRAIN BY ANTIBODY-ABSORPTION TEST

Test No.	Serum		Absorption		HI titre against 8 units of antigen		
	Against strain	No. of injections [a]	Virus	No. of pellets [b]	JaGAr 01	S-705/64	I-58
101	S-705/64	5	None	–	3 840	3 840	240
			JaGAr 01	1	<15	<15	<15
			I-58	1	30	30	<15
				2	15	15	<15
				4	<15	15	<15

[a] The number of injections per mouse used in the production of the serum.

[b] Each virus pellet corresponded to 6.6 g–7.6 g of infected suckling mouse brain. The chosen number of pellets was used to absorb 3 ml of 1 : 10 serum.

Cross-absorption-HI tests of the 2 immunotypes of JEV and 2 non-JE viruses of group B

The experiments described thus far strongly suggest the existence of at least 2 immunotypes of JEV, with Nakayama-RFVL and possibly other strains possessing the immunological characteristics of both types. Clarke (personal communication, 1958) reported antigenic analyses of viruses of the JE–MVE–WN–SLE subgroup by the antibody-absorption test. It is noteworthy that she employed the Nakayama-RFVL strain for JEV. It is interesting to speculate whether the same degree of antigenic difference would have been observed among these viruses if the above-mentioned 2 immunotypes of JEV had been employed.

The results of cross antibody-absorption tests between JEV and related strains are summarized in Table 10. There is no evidence of a particularly close

TABLE 10

IMMUNOLOGICAL COMPARISON OF THE 2 JEV IMMUNOTYPES WITH 2 OTHER VIRUSES OF THE JE-MVE-WN-SLE SUBGROUP BY THE ANTIBODY-ABSORPTION TEST

Test No.	Serum		Absorbing virus [b]	HI titre against 8 units of antigen				
	Against vir usorstrain	No. of injections [a]		MVE	I-58	Nakayama-RFVL	JaGAr 01	WN Eg101
106, 111	MVE	4	None	3 840	240	960	1 920	1 920
			I-58	120	<20	<20	<20	
			JaGAr 01	120	<20	<20	<20	
110	I-58	6	None	960	480	960	960	480
			MVE	<20	120	60	30	<20
			WN Eg101	60	240	240	120	<20
110	JaGAr 01	5	None	1 920	240	480	1 920	960
			MVE	<20	<20	30	120	20
			WN Eg101	30	20	120	240	<20
102, 106	WN Eg101	5	None		60	240	960	1 920
			I-58		<20	<20	<20	240
			JaGAr 01		<20	<20	<20	60

[a] The number of injections per mouse used in the production of the serum.

[b] Used in the minimum amount required to reduce the homologous antibody titre below 1 : 20.

similarity between either immunotype of JEV and MVE or the WN strain Eg101. In fact, the immunological location of MVE and WN appeared to be much further from either immunotype than the distance between the two immunotypes. When MVE serum was absorbed with I-58 or JaGAr 01 virus, 3% of antibody remained unabsorbed in each case. The largest amount of antibody was found unabsorbed when I-58 serum was absorbed with MVE or WN virus, suggesting that the I-58 is immunologically further from either MVE or WN Eg101, especially from the WN strain, than JaGAr 01 is.

A simplified antibody-absorption test for rapid immunological typing of JEV strains

The results reported above strongly suggest that the antibody-absorption test would be suitable for routine immunological typing of JEV strains. A simplified procedure has been developed for this purpose. A hyperimmune serum prepared against the strain of JEV to be typed was absorbed with a single pellet each of I-58 and JaGAr 01 virus and the homologous antibody titres determined after absorption. Absorption with a larger amount of virus was attempted only when both viruses left an appreciable amount of antibody.

Table 11 shows the results obtained with 11 JEV strains, which seem to indicate quite clearly that all these strains are of the same immunotype. The only exception observed was when the Tengah strain was absorbed with the JaGAr 01 virus. (Detailed information on this case is not presented in Table 11.) The absorption of this serum with a single pellet of JaGAr 01 virus gave a residual homologous titre of 1 : 120. Although the antibody was eventually eliminated by absorbing the serum with increasing amounts of the virus, its titre declined unusually slowly, showing what a wide range of immunological properties can exist even in a single immunotype.

Among the other strains, it was hardly predictable that the H-58 strain would be of the JaGAr 01 immunotype, since the biological properties of this strain closely resembled those of the I-58 strain (Nakayama-NIH immunotype) which had been isolated in the same year (1958) by the same laboratory. The remaining 9 strains were readily typed as the JaGAr 01 immunotype.

DISCUSSION

Ogata (1959) demonstrated the difference between G-1-Late and Nakayama-NIH strains by cross vaccination-challenge experiments in which mice were vaccinated with Nakayama-NIH vaccine and challenged intracerebrally with virus. The homologous virus gave a protection index of 3.3, and the heterologous virus a protection index of 0.8, indicating a clear-cut difference between the two. However, vaccination with the G-1-Late strain failed to protect the mice against either homologous or heterologous challenge. Kobayashi (1959) repeated these experiments using live-virus immunization, and also failed to disclose quantitative differences when mice vaccinated with the G-1-Late vaccine were challenged by the homologous and Nakayama-NIH strain. The difficulties in such experiments are how to standardize the potency of the vaccine and the amount of challenge virus, and how to standardize the virulence of the virus strains in order to allow quantitative comparison.

Mouse intracerebral neutralization tests were also employed by Ogata (1959) and Kobayashi (1959) to investigate the difference between these two strains. Ogata found no significant difference between the two with sera prepared by repeated killed vaccination; Kobayashi obtained a unilateral difference with the Nakayama-NIH sera prepared by live vaccination. The mouse neutralization test has a comparatively wide range of error. More is to be expected of the plaque-reduction neutralization test which has been developed recently. This precise tissue-culture technique has enabled comparative kinetic neutralization studies of JEV strains to be carried out (Oya et al., personal communication, 1962). The results obtained so far by this method are in general agreement with ours.

In the initial part of the present study, haemagglutinating antigens from several strains of JEV were found to be poorly reactive against most group-B antisera. Similar observations had been made by Clarke (1960) concerning the Uganda and Indian strains of WN virus. It would not be appropriate to interpret this phenomenon as indicating significant immunological variation, for the following reasons. Firstly, most of the poorly reactive JE haemagglutinins were found to be more thermoresistant than the reactive haemagglutinins at 40°C and pH 9.0 (Okuno et al., unpublished data, 1962). If a poorly reactive antigen has its antigenic determinants less exposed than a reactive antigen, this could explain the observed correlation with the heat resistance. Secondly, Casals (1964), Clarke (1964) and Hamman et al. (1965, 1966) pointed out that some arbovirus haemagglutinins gave a higher HI titre at a pH which was not necessarily optimum for haemagglutination.

TABLE 11

IMMUNOLOGICAL TYPING OF 11 JEV STRAINS BY ANTIBODY-ABSORPTION TEST

Test No.	Serum	Absorbing virus [a]	HI titre against 8 units of antigen [b]				Immunotype
			Homologous	I-58	Nakayama-RFVL	JaGAr 01	
119	Tengah	None	1 920	120	240	960	
		I-58	120	<15	<15	<15	
		JaGAr 01	<15	<15	<15	<15	(JaGAr 01)
112	GH3/52	None	1 920	960	1 920	3 840	
		I-58	60	≦15	≦15	60	
		JaGAr 01	≦15	<15	<15	<15	JaGAr 01
112	Igaya	None	3 840	240	960	3 840	
		I-58	≦15	<15	<15	15	
		JaGAr 01	<15	<15	<15	<15	JaGAr 01
112	Tanaka	None	1 920	480	960	3 840	
		I-58	15	<15	<15	30	
		JaGAr 01	<15	<15	<15	<15	JaGAr 01
112	Hotta	None	1 920	480	960	960	
		I-58	30	<15	<15	15	
		JaGAr 01	<15	<15	<15	<15	JaGAr 01
112	Yachida	None	3 840	960	1 920	3 840	
		I-58	30	<15	<15	30	
		JaGAr 01	<15	<15	<15	<15	JaGAr 01
118	Mochizuki	None	1 920	240	480	960	
		I-58	15	<15	<15	15	
		JaGAr 01	<15	<15	<15	<15	JaGAr 01
119 / 120	H-58	None	480	480	960	1 920	
		I-58	<15	<15	<15	15	
		JaGAr 01	<15	<15	<15	<15	JaGAr 01
118 / 120	JaGAr 21	None	3 840	480	960	1 920	
		I-58	120	<15	<15	60	
		JaGAr 01	15	<15	<15	<15	JaGAr 01
118	Konishi	None	1 920	240	480	960	
		I-58	15	<15	<15	<15	
		JaGAr 01	<15	ND	ND	<15	JaGAr 01
112	JaTH 166 (Mizushima)	None	7 680	960	3 840	7 680	
		I-58	240	<15	60	120	
		JaGAr 01	15	<15	<15	<15	JaGAr 01

[a] Used in the minimum amount required to reduce the homologous antibody titre below 1 : 15, except in the case described in the text.

[b] ND = not done.

It seems possible that most of the poorly reactive antigens have a lower optimum pH, although this assumption did not explain the whole situation in a limited number of the authors' experiments. Regardless of the origin of serum, whether from naturally infected humans or from experimental animals, it is clear that the evaluation of the results of conventional HI tests has to be made carefully, taking account of the superimposition of the reactivity variations.

From the above points of view, the antibody-absorption test has certain advantages. Firstly, the test does not involve complicated infection processes influenced by various non-immunological factors; besides, the mechanisms of haemagglutination have been better elucidated than those of infection processes. Secondly, since the antibody-absorption test employs hyperimmune sera prepared by repeated immunization, the sera are independent of the variability of strains in evoking antibody response. Clarke (1964) in her later researches increased the sensitivity of the antibody-absorption test by comparing antibody-absorption curves obtained at relatively low concentrations (from 1 g to 8 g per ml of undiluted serum) of brain virus. However, in the present study, the criteria for immunological distinction given in her original report (Clarke, 1960) have been followed; the immunological differences observed must thus be regarded as qualitative in nature, being based upon the plateau of unabsorbed antibody in each test (Clarke, 1964). The only technical disadvantage of the antibody-absorption test in its present form is that the variability in haemagglutinating capacity of absorbing viruses still plays a role, as reported previously (Okuno et al., 1965).

Hammam & Price (1966) have been nearly alone among recent authors in suggesting that JEV strains are more or less homogeneous immunologically. These investigators checked 2 Malayan, 1 Indian, 1 Korean and 2 Japanese strains (including Nakayama) by a modification of Casals' HI test, and failed to discover any significant immunological difference. The disagreement between their results and ours is, however, probably only apparent. Their Nakayama was presumably the RFVL strain which, as we have shown here, is an intermediate immunotype. Since the Yachida strain, isolated in our laboratory in 1958 and later typed as JaGAr 01 as described in this report, was included in their study, it is highly probable that all the other strains were also JaGAr 01 immunotype.

Hale & Lee (1954) suggested the existence of 3 immunotypes of JEV—namely, Type 1 (GH3/52), Type 2 (Nakayama, presumably Nakayama-RFVL), and Type 3 (Tengah and Muar)—based upon the results of CF and neutralization tests. Although they did not employ HI and antibody-absorption tests, their results are in good agreement with ours. It is clear that their Type 1 corresponds to our JaGAr 01 immunotype. Although we eventually characterized the Tengah strain as JaGAr 01 immunotype, there are slight quantitative differences between Tengah and JaGAr 01, whereas the Muar strain revealed characteristics extremely similar to the Tengah in another limited antibody-absorption test. It should be stressed that the I-58 immunotype is much further from the JaGAr 01 immunotype than the Tengah or Muar strain is, in antibody-absorption tests. Accordingly, we would suggest that the establishment of a separate type for the Tengah and Muar strains remains open to question until more strains from various geographical regions have been studied.

It should be emphasized that 17 of the 26 strains of JEV studied, which were isolated during a 30-year period, were found to be of the JaGAr 01 immunotype (see Table 1). Considering further that this immunotype was first isolated in 1935, the same year as the Nakayama strain, and considering the frequency of its isolation, it would seem to be justified to suggest that this immunotype is the currently prevalent strain in Japan, and possibly throughout Asia. Nevertheless, the Nakayama-NIH strain has been exclusively employed for JEV vaccine in Japan since 1954, because of the ease with which it can be handled.

Little is known about possible immunological variations of JEV caused by repeated passages through experimental animals. In a previous paper, Okuno et al. (1965) found that 182 passages of the G-1 strain through adult mouse brains seemed to have some effect on certain biological properties. As mentioned above, the conventional HI test failed to show noticeable differences between the G-1-Early and G-1-Late strains. This observation is in line with the findings of Clarke (1960) and Hammam et al. (1965) on strains of WN and yellow fever virus. It should be noted, however, that the antibody-absorption tests reported here revealed a slight but definite shift in the G-1-Late strain towards the I-58 immunotype. This probably indicates the lack of an absolute stability of immunological characteristics, although these are relatively stable compared with biological properties. Could one, perhaps, explain the difference between Nakayama-NIH (I-58 immunotype) and Nakayama-Yakken (JaGAr 01 im-

munotype) on the basis of a shift in properties similar to that which occurred with the G-1 strain? In any event, it is noteworthy that the Nakayama-RFVL strain is believed to have undergone the least number of mouse passages of the three Nakayama sublines, while it seems to retain intermediate immunological properties.

Clarke (personal communication, 1958) analysed the antigenic relationships among the JE–MVE–WN–SLE subgroup by antibody-absorption tests, and found JEV and MVE to be particularly closely related, with SLE relatively far removed from the others. This relationship is not essentially altered if 2 immunological variants of JEV—JaGAr 01 and I-58—are considered. The I-58 strain appears to be further from WN than the JaGAr 01 is. The sharing of a common antigen among group-B arboviruses has become a well-established fact since it was first reported by Casals & Brown (1954). It is highly probable that viruses of a certain antigenic subgroup, or possibly all the group-B viruses, are derived from a single ancestral virus strain. One of the main mechanisms yielding such a variety of different strains might be recombination, while the results obtained here with the G-1 strain suggest that processes of host-mediated variation may also play an important role.

RÉSUMÉ

Les caractéristiques immunologiques de 26 souches du virus de l'encéphalite japonaise (JE) isolées au Japon et en Malaisie, entre 1935 et 1966, ont été étudiées principalement au moyen d'une variante de la réaction d'inhibition de l'hémagglutination: l'épreuve d'absorption des anticorps. On a également utilisé les épreuves IH et de fixation du complément classiques.

Au cours des réactions IH classiques, 5 souches sur les 20 soumises à l'épreuve ont donné des titres uniformément faibles à l'égard des antisérums de divers arbovirus du groupe B. Il semble ressortir des épreuves croisées pratiquées entre des souches de faible titre et d'autres qu'il existe une différence de teneur en hémagglutinine entre les diverses souches. L'épreuve d'absorption des anticorps s'est révélée apte à mettre en évidence des variations immunologiques authentiques, sans intervention défavorable des fluctuations de la réactivité des diverses souches.

Grâce à l'épreuve d'absorption des anticorps, il a été possible de démontrer l'existence de deux immunotypes de virus JE. L'immunotype I-58 comprend deux souches dont l'une, Nakayama-NIH, a été isolée en 1935 à Tokyo et l'autre, I-58, à Okayama dans l'ouest du Japon, en 1958. Les deux souches donnent des titres relativement faibles avec la plupart des antisérums. On a pu montrer que 17 souches isolées au cours des trente dernières années appartiennent à l'immunotype JaGAr 01. Etant donné le nombre important de souches qu'englobe cet immunotype, et la longue période sur laquelle elles ont été isolées, il paraît probable qu'il s'agit de l'immunotype prévalent au Japon et peut-être dans l'ensemble de l'Asie. Il convient toutefois de mentionner que les vaccins contre l'encéphalite japonaise fabriqués dans trois pays d'Asie appartiennent tous à l'immunotype I-58. La souche Nakayama-RFVL présentait les caractéristiques des deux immunotypes en question.

Bien qu'ils soient moins nets, les résultats des épreuves de fixation du complément confirment, dans l'ensemble, ceux de la réaction IH. Selon les auteurs, les souches Tengah et Muar ne correspondent exactement ni à l'un ni à l'autre des immunotypes. Mais, étant donné que l'anticorps spécifique de ces souches est éliminé par de très hautes concentrations du virus JaGAr, on les a provisoirement classées toutes les deux dans ce dernier groupe.

Lorsqu'on compare le virus JE avec des souches d'arbovirus apparentés — encéphalite de Murray Valley (MVE) et West Nile (WN) Eg 101 — par la méthode de l'absorption des anticorps, on constate que l'immunotype I-58 diffère davantage de MVE et de WN que l'immunotype JaGAr 01.

Les réactions d'absorption des anticorps pratiquées sur les souches G-1 Early et G-1 Late ont montré qu'une souche de virus pouvait changer d'immunotype après plusieurs passages sur la souris, ce qui explique peut-être que la situation de la souche Nakayama ne paraisse pas très claire. Celle-ci comporte, en effet, trois sous-populations: Nakayama-NIH, qui appartient à l'immunotype I-58; Nakayama-Yakken, qui appartient à l'immunotype JaGAr 01; et Nakayama-RFVL qui possède les caractéristiques des deux immunotypes.

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