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Antigenic Analysis of Japanese Encephalitis Virus by Using Monoclonal Antibodies

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Hybridoma cells were produced by fusing P3X63Ag8.653 mouse myeloma cells with spleen cells from BALB/c mice immunized with Japanese encephalitis (JE) virus, Nakayama-RFVL strain. The resulting 26 clones produced hemagglutination inhibition antibodies against the homologous strain. The hemagglutination inhibition reactivity of each clone was tested against six flaviviruses: JE, Murray Valley encephalitis (MVE), Egypt 101 strain of West Nile (WN), St. Louis encephalitis (SLE), Russian spring summer encephalitis, and dengue type 1. The 26 monoclonal antibodies fell into four groups: 14 JE species-specific antibodies, 6 antibodies reactive to JE and MVE viruses, 3 antibodies to three or four viruses in the JE-MVE-WN-SLE subgroup, and 3 antibodies to all six flaviviruses. Furthermore, antigenic comparison of 27 strains of JE virus was carried out by using five JE species-specific monoclonal antibodies. Of these, 24 strains were isolated in various parts of Japan, and 3 strains came from Southeast Asia. In reactivity, the 27 strains were classified into at least four antigenic groups. The results showed that the Nakayama-Yakken strain is a mutant strain which lacks the Nakayama strain-specific antigen and that the recently isolated strains are immunologically different from Nakayama and JaGAr 01 strains. One clone (NARMA 13) produced a JE species-specific antibody which showed almost the same titer against 26 JE virus strains, whereas one clone (NARMA 5) produced a Nakayama strain-specific antibody which reacted only to the Nakayama-RFVL and Nakayama-Yoken strains.

Japanese encephalitis (JE) virus belongs to the genus *Flavivirus* according to its physicochemical properties. The virus has also been classified as a group B arbovirus by its epidemiological and serological properties.

Since Casals and Brown (4) in 1954 showed cross-reactivity of arboviruses by using the hemagglutination inhibition (HI) test, many arboviruses have been serologically classified into several groups by use of the HI test, the complement-fixation test, or the neutralization test. Flaviviruses (group B arboviruses) revealed marked cross-reactivity to each other against conventional hyperimmune animal antisera. Particularly, JE, Murray Valley encephalitis (MVE), West Nile (WN), and St. Louis encephalitis (SLE) viruses have exhibited close antigenic relationships under various serological procedures (2, 18). However, the HI test has been the best method for the demonstration of antigenic relationships among the members of flaviviruses. Moreover, Clarke (5) has applied the antibody absorption technique of the HI test to the analysis of relationships among certain flaviviruses.

On the other hand, Hale and Lee (8), in 1954, observed that the properties of six strains of JE virus isolated in Malaya showed dissimilarity to those of the Nakayama strain. Since then, comparative studies of the antigenic structures of JE virus have been performed by many investigators (1, 10, 14, 16, 19).

It is considered that the monoclonal antibody initially developed by Köhler and Milstein (15) is a powerful tool in the antigenic analysis of flaviviruses. Monoclonal antibodies against flaviviruses have been prepared for analysis of dengue virus serotypes by Dittmar et al. (7), for study of the phenomenon of antibody-dependent enhancement of replication with the use of WN virus by Peiris et al. (17), and for antigenic analysis of tick-borne encephalitis virus by Heinz et al. (11).

In this report, we describe the characterization of 26 monoclonal antibodies against the Nakayama-RFVL strain of JE virus and the results of the antigenic analysis of 27 JE virus strains by using 5 JE species-specific monoclonal antibodies.

MATERIALS AND METHODS

Viruses. Twenty-seven strains of JE virus and the other five flaviviruses, MVE, WN Egypt 101, SLE, Russian spring summer encephalitis (RSSE), and dengue type 1 (DEN-1), were used. The JE virus strains used in this study are listed in Table 1. Of the three Nakayama strains, the Nakayama-RFVL strain has been maintained at the Rockefeller Foundation Virus Laboratories, New York, the Nakayama-Yoken strain has been maintained at the National Institute of Health, Tokyo, Japan, and the Nakayama-Yakken strain has been maintained at the Ministry of Agriculture and Forestry, Tokyo, Japan. Ten strains of JE virus, Nakayama-Yakken, Kalinina, G-1 late, Sekiya, Mochizuki, JaGAr 02, Mie 44-1, Kumamoto 80679, Muar, and 691004, and the other five flaviviruses were provided by the National Institute of Health, Japan. The other 17 strains of JE virus have been maintained in our laboratories.

Preparation of mouse myeloma cells. P3X63Ag8.653 (Ag8.653) mouse myeloma cells were used. Cells which do not synthesize immunoglobulin were produced by Kearney et al. (13) and were provided by the Salk Institute, La Jolla, Calif. The cells were maintained in RPMI 1640 medium with 10% fetal calf serum and 100 μM 8-azaguanine at 37°C in a 5% CO_2 incubator. The medium was exchanged on the day

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TABLE 1. JE virus strains used

JE virus strain[a]	Isolation			Passage
	Year	Place	Source	
Nakayama-RFVL	1935	Tokyo	Human CSF[b]	numerous
Nakayama-Yoken	1935	Tokyo	Human CSF	numerous
Nakayama-Yakken	1935	Tokyo	Human CSF	92
Kalinina	1935	Tokyo	Human brain	64
G-1 late	1949	Tokyo	Human brain	196
Sekiya	1952	Tokyo	Human brain	8
Mochizuki	1953	Okayama	Human brain	7
JaGAr 01	1959	Gunma	Mosquito	5
JaGAr 02	1959	Gunma	Mosquito	14
Nishizono	1964	Fukuoka	Human brain	5
JaFAr 401465	1965	Fukuoka	Mosquito	2
JaFS 01	1965	Fukuoka	Swine blood	2
Hatano 65	1965	Kyoto	Human brain	4
Kamiyama	1966	Fukuoka	Human brain	5
Sasazaki	1966	Tokyo	Human brain	5
Mie 44-1	1969	Mie	Mosquito	8
Fukuoka 7101	1971	Fukuoka	Swine blood	4
Fukuoka 7202	1972	Fukuoka	Swine blood	3
Fukuoka 7309	1973	Fukuoka	Swine blood	3
Fukuoka 7311	1973	Fukuoka	Swine blood	3
Fukuoka 7452	1974	Fukuoka	Swine blood	3
Fukuoka 7463	1974	Fukuoka	Swine blood	3
Fukuoka 7506	1975	Fukuoka	Swine blood	3
Kumamoto 80679	1979	Kumamoto	Mosquito	5
Muar	1952	Singapore	Human brain	24
Chiang Mai	1964	Thailand	Human brain	10
691004	1969	Sri Lanka	Human brain	11

[a] Twenty-four strains were isolated in Japan, and three strains were isolated in Southeast Asia.

[b] CSF, Cerebrospinal fluid.

before fusion, and the cells were taken at the exponential growth phase for fusion.

Immunization of mice. Live and inactivated vaccines of Nakayama-RFVL strain were prepared. A supernatant (10^{-2} dilution) of infected suckling mouse brain suspension with 0.15 M saline solution was inoculated intracranially into 3-day-old mice. The brains were harvested at the acme of the illness when one-third of the mice died, suspended in 0.15 M saline solution, and homogenized with a Waring blender. After centrifugation at 2,500 rpm for 10 min at 4°C, the supernatant was divided into two parts. One was inactivated with Formalin at 0.05% of the final concentration and was used as the inactivated vaccine.

BALB/c 6-week-old mice were injected intraperitoneally with inactivated vaccine on days 1 and 3, followed by live vaccine diluted to 10^{-3} with saline solution on days 10, 15, and 20. Some of the mice were bled by heart puncture 10 days after the last immunization, and the serum was stored at −20°C as the immune serum. The other mice were boostered with an intravenous injection of the live vaccine 3 days before spleen cells were removed for fusion.

Preparation of spleen cells. Spleens removed from two immune mice were finely cut and crushed on a 200-mesh screen, while being repeatedly washed with Dulbecco modified Eagle medium without serum. The cell suspension was centrifuged at 1,000 rpm for 5 min, and the supernatant was discarded. This procedure was repeated twice, and the pellet was then resuspended at a cell concentration of 1×10^7 to 1.5×10^7 lymphocytes per ml.

Fusion procedure. Of the lymphocyte suspension, 20 ml was mixed with 20 ml of Ag8.653 cell suspension at a cell ratio of 10:1; the cells were pelleted together in a conical tube. The supernatant was aspirated, and the pellet was dispersed by tapping it. Then, 1.0 ml of 45% polyethylene glycol (PEG 4000; Sigma Chemical Co.) in Dulbecco modified Eagle medium was added. After 7 min at 37°C, the cells were gently suspended in 40 ml of Dulbecco modified Eagle medium and centrifuged at 1,000 rpm for 5 min. The pellet was then evenly resuspended at a concentration of 1×10^6 Ag8.653 cells per ml in HT medium. HT medium consisted of RPMI 1640 with 20% fetal calf serum, 2mM 1-glutamine, 10^{-4} M hypoxanthine, 1.6×10^{-5} M thymidine, 100 U of penicillin G per ml, and 100 μg of streptomycin per ml. Thereafter, the suspension was gently distributed into 96-well microplates at 50 μl per well and was incubated at 37°C in a 5% CO_2 incubator. The next day, 50 μl of the HT medium containing 0.8 μM aminopterin was added. At 6 days later, 100 μl of HT medium containing 0.4 μM aminopterin was added. When clones appeared macroscopically between 10 and 20 days, the supernatant fluids were screened for the production of HI antibody against the homologous antigen. Antibody-producing hybridoma cells were cloned by limiting dilution. Thymocytes were prepared from BALB/c mice by the same method for the spleen cell suspension described previously and were used as feeder cells. The cells were suspended at a concentration of 1×10^7 to 2×10^7/ml in HT medium, and 100 μl of the suspension was placed into each well of 96-well plates. The suspensions of hybridoma cells at a concentration of six cells per ml were prepared in HT medium by the limiting dilution method, and 100 μl of the suspension was added to each well of the plates.

Colonies appeared in 10 to 14 days after seeding. The supernatants from single-colony wells were screened and the antibody-producing hybridoma cells were recloned as previously described. These clones were subsequently expanded into mass culture and frozen to assure retension.

For production of ascitic fluids, BALB/c mice were injected intraperitoneally with 0.5 ml of Pristane (Aldrich) 7 to 9 days before the intraperitoneal injection of 10^7 hybridoma cells. The ascitic fluids containing antibody and hybridoma cells were obtained after about 2 weeks.

Determination of antibody class and subclass. Monoclonality of hybridoma antibodies was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and micro-Ouchterlony method. The hybridoma cells were cultured in RPMI 1640 medium, supplemented with 1% gamma globulin-free fetal calf serum (GIBCO). After being concentrated by the 50% saturated ammonium sulfate precipitation method and undergoing affinity chromatography with DEAE Affi-Gel Blue (Bio-Rad), these supernatants were analyzed by 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the micro-Ouchterlony method against rabbit antisera reacting with mouse specific immunoglobulins: immunoglobulin G (IgG), IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, κ, and λ chains (Miles).

Analysis of chromosomes in hybridomas. The karyotype was analyzed at the metaphase stage of mitotic cells by adding colchicine (GIBCO) to give a final concentration of 0.07 μg/ml to the culture medium on day 2 or 3. At 2 h later, the cells were harvested, fixed in methanolacetic acid (3:1) and stained by the Giesma method.

HI tests. HI tests were performed by the method of Clarke and Casals (6). The antigens were prepared by the sucrose-acetone extraction method. A dilution of each antigen was made in cold bovine albumin-borate-saline to give an estimated 8 U of hemagglutinin per 0.2 ml. Immune mouse serum, culture supernatants, and ascitic fluids of hybrido-

mas were treated by the acetone extraction method for removal of nonspecific inhibitors. Erythrocytes were prepared from 1-day-old chickens and were stored at 10% suspension in dextrose-gelatin-Veronal. The stock cells were diluted 1:24 in the various virus adjusting diluents. All tests were carried out on plates.

RESULTS

Hemagglutination titers and optimal pH values of antigens. All of the antigens showed high hemagglutination titers, i.e., 1:4,800 to 1:80,000, with the exception of RSSE virus antigen which was 1:800. In regard to the JE virus antigens, the strains which underwent many passages in mice had a tendency to show lower optimal pH values than those which underwent only a few passages. Furthermore, the Nakayama strains had different optimal pH values: Nakayama-RFVL at pH 6.5, Nakayama-Yoken at pH 6.3 and Nakayama-Yakken at pH 6.4.

Production of antibody-secreting hybridomas. Twenty-six cell lines were obtained, and the resulting hybridomas were called NARMA, i.e., Nakayama-RFVL monoclonal antibody, 1 to 26.

Monoclonality and determination of classes and subclasses of immunoglobulin of the hybridomas. To characterize the monoclonality of the hybridoma antibodies, concentrated supernatants of 26 clones were tested by the micro-Ouchterlony method. Of these, all four IgM clones belonged to the species-specific antibody producing group, moreover, they had a tendency to strain-specificity against the homologous strain. Of IgG clones, 21 showed species-, subgroup-, or genus-specific properties. One IgA clone was subgroup-specific. In addition, the IgG antibodies demonstrated the clear, single band in the position expected for mouse IgG by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Karyotypic analysis. The chromosome numbers of the representative eight hybridomas and the Ag8.653 cells are shown in Table 2. The mean range of the chromosome numbers of the hybridomas was 86.6 to 95.4.

Reactivity of the monoclonal antibodies against six flaviviruses. To clarify the immunological characterization of these 26 monoclonal antibodies, the HI reactivity of each was tested against six flavivirus antigens. Table 3 shows the results of the HI test with anti-Nakayama-RFVL hyperimmune mouse serum and ascitic fluids produced by the hybridomas. The HI titer of immune mouse serum showed cross-reactivity to a marked degree. The antibodies produced by the 26 hybrid cell clones fell into four groups:

(i) Fourteen clones (NARMA 1 to 14) produced JE species-specific antibodies. NARMA 1 to 12 antibodies reacted specifically to the homologous antigen. NARMA 13 antibody reacted with the HI titer of 1:2,048 against MVE antigen and NARMA 14 antibody reacted with the titer of 1:2,048 against both WN and SLE antigens as compared with that of the homologous antigen.

(ii) Six clones (NARMA 15 to 20) produced antibodies which reacted to the homologous and MVE viruses. The titer against MVE virus antigen showed 8 to 1:128 as compared with that of the homologous antigen.

(iii) Three clones (NARMA 21 to 23) produced antibodies reactive to three or four virus antigens in the JE-MVE-WN-SLE subgroup.

(iv) Three clones (NARMA 24 to 26) produced antibodies cross-reactive to all six flaviviruses, JE, MVE, WN, SLE, RSSE, and DEN-1.

Reactivity of the monoclonal antibodies against four strains of JE virus. Fourteen monoclonal antibodies, NARMA 1 to 14, were tested against the four JE virus strains, Nakayama-RFVL, Nakayama-Yoken, JaGAr 01, and Kamiyama. They consisted of the following antibodies (Table 4).

(i) NARMA 5 and 6 showed almost the same titer against the Nakayama-RFVL and Nakayama-Yoken strains, but did not react to the other strains. These antibodies showed Nakayama strain-specificity.

(ii) NARMA 1, 2, and 8 showed 1:1 to 1:4 against the Kamiyama strain as compared with that of the Nakayama-RFVL strain, and lower or negative against the JaGAr 01 strain.

(iii) NARMA 4 had a titer of 1:64 against the JaGAr 01 and Kamiyama strains as compared with that of the Nakayama-RFVL strain.

(iv) NARMA 10 to 14 showed almost the same titer against the four strains and the antibodies produced by the remaining three clones showed the titers of intermediate type. They were regarded as species-specific antibody.

Classification of 27 JE virus strains by the HI test by using five monoclonal antibodies. Five monoclonal antibodies (NARMA 1, 4, 5, 8, and 13) characterized by the different reactivities were used in this test. The results are shown in Table 5. According to the pattern of reactivity against the antibodies, the 27 strains are classified into the following groups:

(i) The Nakayama-RFVL and Nakayama-Yoken strains showed high titers against five antibodies. On the other hand, the Nakayama-Yakken strain showed the same titer against NARMA 4, lower against NARMA 1, 8, and 13 as compared with the Nakayama-RFVL strain, and negative against NARMA 5. Although Nakayama-Yakken strain is derived from the same origin as Nakayama-RFVL and Nakayama-Yoken strains, an antigenic difference was observed between the Nakayama-Yakken strain and the other two Nakayama strains. Thus, the Nakayama-RFVL and Nakayama-Yoken strains are referred to as group I-1, and the Nakayama-Yakken strain is referred to as group I-2.

(ii) Four strains, Kalinina, G-1 late, JaGAr 01, and JaGAr 02, showed almost the same titer against NARMA 13, lower against NARMA 1 and 4 as compared with the Nakayama-RFVL strain, and negative against NARMA 5 and 8. These four strains are referred to as group II.

(iii) Nineteen strains, Sekiya, Mochizuki, Nishizono, Ja-

TABLE 2. Karyotype of hybridomas[a]

Cell line	Chromosome number	
	Range	Mean ± SD
Parents		
Mouse lymphocyte		40
P3X63Ag8.653	55–68	61.1 ± 5.6
Hybridomas		
NARMA 1	78–110	94.4 ± 11.6
4	82–99	91.3 ± 8.0
5	84–101	91.9 ± 7.4
8	79–113	94.7 ± 14.6
13	77–111	92.7 ± 13.4
15	78–97	86.6 ± 8.4
23	79–113	94.7 ± 14.6
24	84–125	95.4 ± 10.0

[a] The karyotype of myeloma cell line P3X63Ag8.653 was counted before cell fusion. The karyotype of hybridomas was counted at 3 to 4 months after cell fusion.

TABLE 3. HI titers of hybridoma ascitic fluids against flaviviruses[a]

Hybridoma	Immunoglobulin class	HI titers					
		JE[b]	MVE	WN[c]	SLE	RSSE	DEN-1
Immune mouse serum[d]		2,560	2,560	2,560	640	320	320
NARMA 1	G1 κ	81,920	·	·	·	·	·
2	G2a κ	40,960	·	·	·	·	·
3	G1 κ	40,960	·	·	·	·	·
4	M κ	40,960	·	·	·	·	·
5	M κ	20,480	·	·	·	·	·
6	M κ	10,240	·	·	·	·	·
7	G2a κ	10,240	·	·	·	·	·
8	M κ	5,120	·	·	·	·	·
9	G2a κ	2,560	·	·	·	·	·
10	G2a κ	2,560	·	·	·	·	·
11	G2b κ	2,560	·	·	·	·	·
12	G2a κ	1,280	·	·	·	·	·
13	G3 λ	20,480	10	·	·	·	·
14	G2a κ	20,480	·	10	10	·	·
15	G1 κ	163,840	10,240	·	·	·	·
16	G3 κ	10,240	81,920	·	·	·	·
17	G2a κ	10,240	1,280	·	·	·	·
18	G1 κ	2,560	20	·	·	·	·
19	G1 κ	1,280	160	·	·	·	·
20	G2a κ	640	40	·	·	·	·
21	G1 κ	2,560	160	20	·	·	·
22	G1 κ	160	40	·	20	·	·
23	A κ	1,280	20,480	20,480	2,560	·	·
24	G2a κ	81,920	327,680	327,680	327,680	40,960	20,480
25	G1 κ	5,120	81,920	81,920	40,960	20,480	10,240
26	G2a κ	5,120	40,960	40,960	20,480	2,560	1,280

[a] The titer is expressed as the reciprocal of dilution. ·, Negative or less than 1:10.
[b] JE, Nakayama-RFVL strain.
[c] WN, Egypt 101 strain.
[d] Immune mouse serum, anti-Nakayama-RFVL immune mouse serum.

FAr 401465, JaFS 01, Hatano 65, Kamiyama, Sasazaki, Mie 44-1, Fukuoka 7101, Fukuoka 7202, Fukuoka 7309, Fukuoka 7311, Fukuoka 7452, Fukuoka 7463, Fukuoka 7506, Kumamoto 80679, Chiang Mai, and 691004, showed almost the same titer against NARMA 1 and 13 as the Nakayama-RFVL strain, and reacted to NARMA 4 and 8 to various degrees, but not to NARMA 5. However, the titer of the 691004 strain against NARMA 4 and 8 was nearly the same as that of the Nakayama-RFVL strain and higher than that of the other strains in this group. In addition, this strain had a higher titer against NARMA 4 than that against NARMA 8, whereas the remaining 18 strains were higher against NARMA 8 than against NARMA 4. Therefore, the 691004 strain may be immunologically different from the other 18 strains. These 18 strains are referred to as group III-1, and the 691004 strain is referred to as group III-2.

(iv) The Muar strain, referred to as group IV, showed almost the same titer against NARMA 13 and a lower titer against NARMA 1 as compared with the Nakayama-RFVL strain, but did not react to NARMA 4, 5, and 8.

NARMA 13 is regarded as a typical JE species-specific antibody, because this antibody demonstrated almost the same titer against all JE virus strains used in this study except for the Nakayama-Yakken strain. In contrast, NARMA 5 is a Nakayama strain-specific antibody, because this reacted only to the Nakayama-RFVL and Nakayama-Yoken strains but not to other strains. The Nakayama-Yakken strain is immunologically distinguished from the other two Nakayama strains, especially in its lack of reaction to NARMA 5. It is suggested that the Muar strain is the most antigenically different from the Nakayama-RFVL strain.

Table 6 indicates the relationship between the antigenic differences and the year of isolation of 27 JE virus strains. In Japan, 24 strains isolated from different areas between 1935 and 1979 fell into at least three antigenic groups, I, II, and III-1. The Nakayama strains (group I), the oldest JE virus strain, isolated in Tokyo in 1935, was different from the other strains. Moreover, there was an antigenic difference between the Nakayama-Yakken strain and the other two Nakayama strains. There were four virus strains (group II) which shared the antigenic characteristics of the JaGAr 01 strain: Kalinina and G-1 late in Tokyo, JaGAr 01 and JaGAr 02 in Gunma. These four strains were isolated during 1935 to 1959. There were 17 strains (group III-1) which shared the antigenic characteristics of the Kamiyama strain: Sekiya in Tokyo, 1952; Mochizuki in Okayama, 1953; and 15 strains isolated in various areas of Japan after 1964. The recently prevalent strains in Japan belong to group III-1.

Geographically, three strains in Southeast Asia, Muar, Chiang Mai, and 691004, were antigenically different from each other. Of these three strains, the Chiang Mai strain shared the antigenic characteristics of the recently isolated strains in Japan.

DISCUSSION

By using cell fusion technology, 26 hybrid cell lines secreting HI antibodies against the Nakayama-RFVL strain of JE virus were produced.

Fourteen of these antibodies, NARMA 1 to 14, reacted only to JE virus and showed varied reactive characterization in the HI test against four JE virus strains: Nakayama-RFVL, Nakayama-Yoken, JaGAr 01, and Kamiyama. NARMA 13 and 14 were typical species-specific antibodies. On the other hand, NARMA 5 and 6 were strict Nakayama strain-specific antibodies. The other antibodies showed intermediate reactivities. The Nakayama strain, isolated in 1935, was the first strain of JE virus. Since then, the strain has been maintained in several different laboratories by prolonged mouse brain passages. The Nakayama-RVFL strain was kept in the Rockefeller Foundation Virus Laboratories and is now in the Yale Arbovirus Research Unit, New Haven, Conn., and is a reference strain of JE virus. The Nakayama-Yoken strain has been maintained in the National Institute of Health, Japan, and has been used as a vaccine strain for humans. The optimal pH of hemagglutination of the Nakayama-RFVL antigen was 6.5, whereas that of the Nakayama-Yoken antigen was 6.3. In spite of their different histories and different optimal pH values of hemagglutination these two Nakayama antigens showed almost the same titer against NARMA 1 to 14. These findings indicate that Nakayama-RFVL strain and Nakayama-Yoken strain are antigenically identical. Three strains of JE virus, Nakayama, JaGAr 01 and Kamiyama, were used to compare the reactivity of the 14 antibodies. NARMA 5 and 6 reacted only against the Nakayama antigen, and NARMA 8 reacted to the Nakayama and Kamiyama antigens, but not to the JaGAr 01 antigen. These reveal clear evidence of antigenic dissimilarity of the three JE virus strains.

It has been known that JE, MVE, WN, and SLE viruses form a subgroup. Of these, the first three are most closely related (5). The antibodies produced by six clones, NARMA 15 to 20, reacted to JE and MVE viruses, but did not to other viruses. These findings seem to indicate that JE virus is the most closely related to MVE virus.

Three monoclonal antibodies, NARMA 21 to 23, reacted to three or four viruses of the JE-MVE-WN-SLE subgroup. NARMA 23 reacted to JE, MVE, WN, and SLE viruses and the antibody titers against the heterologous viruses were higher than that of the homologous virus. It seems that NARMA 23 was the representative subgroup-specific antibody. NARMA 21 reacted to JE, MVE, and WN viruses; NARMA 22 reacted to JE, MVE, and SLE viruses. These findings show that the antigenic determinants of the viruses of the subgroup are complicated.

Furthermore, three monoclonal antibodies, NARMA 24 to 26, reacted to all the flaviviruses tested and the antibody titers showed no great difference. Although RSSE and DEN-1 viruses are distantly related to JE virus, they showed high titers against NARMA 24 to 26, which seem to be typical genus-specific antibodies.

The antigenic variation among JE virus strains has been observed by many investigators since it was first suggested by Hale and Lee (8). However, the reports of various investigators have been inconsistent and frequently conflict-

TABLE 4. HI titers of hybridoma ascitic fluids against JE virus strains[a]

Hybridoma	HI titers			
	Nakayama-RFVL	Nakayama-Yoken	JaGAr 01	Kamiyama
Immune mouse serum[b]	2,560	2,560	2,560	2,560
NARMA 1	81,920	40,960	2,560	40,960
2	40,960	40,960	1,280	10,240
3	40,960	5,120	1,280	2,560
4	40,960	40,960	640	640
5	20,480	20,480	·	·
6	10,240	10,240	·	·
7	10,240	2,560	1,280	2,560
8	5,120	5,120	·	5,120
9	2,560	1,280	640	160
10	2,560	2,560	1,280	1,280
11	2,560	2,560	1,280	640
12	1,280	1,280	320	320
13	20,480	40,960	40,960	10,240
14	20,480	10,240	10,240	5,120

[a] The titer is expressed as the reciprocal of dilution. ·, Negative or less than 1:10.

[b] Immune mouse serum, anti-Nakayama-RFVL immune mouse serum.

TABLE 5. HI titers of hybridoma ascitic fluids against 27 JE virus strains[a]

Antigen	HI titers				
	NARMA 13	NARMA 1	NARMA 4	NARMA 8	NARMA 5
Nakayama-RFVL	20,480	81,920	40,960	5,120	20,480
Nakayama-Yoken	40,960	40,960	40,960	5,120	20,480
Nakayama-Yakken	2,560	1,280	20,480	20	·
Kalinina	10,240	640	5,120	·	·
G-1 late	10,240	1,280	1,280	·	·
JaGAr 01	40,960	2,560	640	·	·
JaGAr 02	20,480	5,120	640	·	·
Sekiya	10,240	20,480	40	1,280	·
Mochizuki	20,480	20,480	640	2,560	·
JaFAr 401465	40,960	20,480	20	320	·
JaFS 01	40,960	20,480	20	320	·
Hatano 65	20,480	20,480	40	1,280	·
Nishizono	20,480	20,480	160	2,560	·
Kamiyama	20,480	40,960	640	5,120	·
Sasazaki	20,480	40,960	320	5,120	·
Mie 44-1	10,240	40,960	640	1,280	·
Fukuoka 7101	20,480	40,960	320	5,120	·
Fukuoka 7202	10,240	40,960	640	2,560	·
Fukuoka 7309	10,240	40,960	640	1,280	·
Fukuoka 7311	10,240	40,960	160	1,280	·
Fukuoka 7452	10,240	40,960	640	1,280	·
Fukuoka 7463	10,240	40,960	160	1,280	·
Fukuoka 7506	20,480	40,960	1,280	5,120	·
Kumamoto 80679	10,240	40,960	320	2,560	·
Muar	40,960	40	·	·	·
Chiang Mai	20,480	40,960	20	640	·
691004	20,480	20,480	81,920	5,120	·

[a] The titer is expressed as the reciprocal of dilution. ·, Negative or less than 1:10.

TABLE 6. Serological classification of JE virus strains used

Yr of isolation	Group I		Group II	Group III		Group IV
	1	2		1	2	
1935	Nakayama-RFVL					
	Nakayama-Yoken					
		Nakayama-Yakken				
			Kalinina			
1949			G-1 late			
1952				Sekiya		Muar
1953				Mochizuki		
1959			JaGAr 01			
			JaGAr 02			
1964				Nishizono		
				Chiang Mai		
1965				JaFAr 401465		
				JaFS 01		
				Hatano 65		
1966				Kamiyama		
				Sasazaki		
1969				Mie 44-1	691004	
1971				Fukuoka 7101		
1972				Fukuoka 7202		
1973				Fukuoka 7309		
				Fukuoka 7311		
1974				Fukuoka 7452		
				Fukuoka 7463		
1975				Fukuoka 7506		
1979				Kumamoto 80679		

ing. In the present study, antigenic comparison of the 27 JE virus strains, 24 strains of which were isolated in Japan and 3 strains in Southeast Asia between 1935 and 1979, was carried out by the HI test by using five monoclonal antibodies characterized by the different reactivities. These 27 strains could be classified into at least four major antigenic groups.

In group I, Nakayama-RFVL and Nakayama-Yoken strains are antigenically identical, but the Nakayama-Yakken strain is a mutant which lacks the Nakayama strain-specific components. This indicates that the immunological characteristics of JE virus may change during the mouse brain passages, although they are relatively stable. The four strains of group II are distinguished from other strains. This group was isolated during 1935 to 1959. This observation indicates that at least two immunologically distinct strains existed in Japan in 1935. The 18 strains of group III-1 have been frequently isolated in different areas of Japan since they were first isolated in Tokyo in 1952. Accordingly, it is strongly suggested that the currently prevalent strain in Japan belongs to this group. The 691004 strain of group III-2 may be antigenically different from the other 18 strains in its reactivities. The Muar strain of group IV is the most immunologically distinct from the Nakayama-RFVL strain.

Previous studies have shown that antigenic difference among strains of several arboviruses was related to the geographic origin (3, 5, 9, 10, 12, 20). In regard to the JE virus, however, Hammam and Price (10) have reported that they examined Malayan, Indian, Korean, and Japanese strains by HI test and failed to discover any significant immunological differences. The present study clearly shows that at least three antigenically distinct strains of JE virus have existed in Japan over a span of more than 40 years. However, the mechanisms of antigenic variation and dissemination are obscure. The recently isolated strains differed from Nakayama or JaGAr 01 strains. Nevertheless, the Nakayama-Yoken strain has been exclusively employed for a JE virus vaccine, and JaGAr 01 strain has been used as a diagnostic antigen in Japan. The question arises as to whether these two strains are suitable as the materials of a vaccine or diagnostic antigen at present. The three strains in Southeast Asia, Muar, Chiang Mai, and 691004, were antigenically different from each other. The Chiang Mai strain shared the antigenic characteristics of the recently isolated strains in Japan. However, it would not be appropriate to conclude that the antigenic difference is based on geographic origin, because the strains used in this study are a small part of prevalent strains of JE virus, and there are at least three antigenic differences among the strains in Japan. To resolve this problem, it will be necessary to do further studies with more strains of JE virus isolated in various areas.

The monoclonal antibody has remarkable advantage over the immune animal serum heretofore in use. At present, accurate antigenic analysis and identification of the isolate can be done by preparation of the monoclonal antibodies from strain-specificity to genus-specificity. Monoclonal technology appears to provide more detailed knowledge of the antigenic structure of flaviviruses than any other techniques so far utilized. In the future, the flavivirus may be classified systematically and practically by use of cell fusion technology. We expect that these monoclonal antibodies will serve in the identification and antigenic analysis of the JE virus and other flaviviruses.

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