

Comparison of Immunological, Biological and Biochemical Properties of Japanese Encephalitis Virus Strains Isolated in Japan and China †

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Abstract : Two Chinese strains of Japanese encephalitis virus (JEV), A2 and P3 were compared with three Japanese strains, Nakayama-NIH, Mie 44-1 and JaGAR-01 by antibody-absorption test and hemagglutination (HA) test. Structural polypeptides were analyzed by polyacrylamide gel electrophoresis. A2 and Nakayama-NIH strains were found to possess specific antigens in addition to common antigens. However, Mie 44-1 and A2 were found to have almost the same antigenicity. Hemagglutinins of A2, P3 and Nakayama-NIH showed optimum pH at 6.4 or lower and stable for heating at 37°C. JaGAR-01 strain possessing optimum pH at 6.8 or higher was unstable for heating. In spite of these biological and immunological differences, analysis of the structural polypeptides by polyacrylamide gel electrophoresis showed no difference.

INTRODUCTION

The evidences of immunological difference of JEV strains have been accumulated. Hale and Lee (1954) firstly reported the existence of immunological variation in JEV. Kobayashi (1959) and Ogata (1959) demonstrated the difference between G-1 and Nakayama-NIH strains by cross protection, hemagglutination-inhibition(HI), complement-fixation (CF) and neutralization tests. Okuno et al. (1968) attempted immunotyping of different strains of JEV by antibody-absorption, hemagglutination-inhibition and complement fixation tests. The results of those investigations showed that two immunotypes could be distinguished among a number of JEV strains isolated in Japan. Not only from the view-point of effective vaccination, an investigation on the existing immunotypes of JEV

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is considered to be important for the epidemiology and ecology of JE. Oda (1976) reported no appreciable antigenic difference among newly isolated strains by neutralizing antibody-absorption test.

The JEV is now prevailing widely in the east and south east parts of Asia. Yet, papers were seldom to deal with the comparison of the properties of JEV strains from the different geographical regions. The present report reveals the results of a comparative study on immunological and biochemical properties of JEV strains isolated in China and Japan.

MATERIALS AND METHODS

Virus strains: All viruses were prepared as 10% emulsions of infected suckling mouse brain in 1/75 M PBS, pH 7.4 containing 2% calf serum. The emulsions were centrifuged at $7,000 \times g$ for 15 minutes in a refrigerated centrifuge and the supernatants were stored at -80°C as virus stocks. The origin and passage histories of virus employed in the study are described in Table 1.

Preparation of hyperimmune sera: Six week old ddY strain of mice were used for immunization. For Mie 44-1 and A2 viruses, mice were immunized intraperitoneally 4 times by a week interval with diluted infected mouse brain suspension except the initial immunization with 0.1% β -propiolactone inactivated vaccine. For Nakayama-NIH virus, all immunization was made with live virus. Mice were bled a week after the last im-

Table 1. Strains of Japanese encephalitis virus

Name of strain	Isolation			Passage histories
	Year	Place	Source	
Nakayama-NIH	1935	Tokyo	Human CSF	Ad multiple Sm 9
JaGAR-01	1959	Gumma	<i>Culex tritaeniorhynchus</i>	Sm 6 Ad 2 Sm 8
Mie 44-1	1969	Mie	<i>Culex tritaeniorhynchus</i>	Sm 5
A2	1949	Beijing	Human brain	Ad 37
P3	1949	Beijing	Human brain	Ad 56

Ad: Adult mouse brain Sm: Suckling mouse brain

CSF: Cerebrospinal fluid

munization, then sera were separated and stored at -20°C .

The antibody-absorption test: The procedures followed essentially those reported by Clarke (1960). Absorbing viruses were obtained from 20% infected suckling mouse brain suspension by centrifugation at $7,000\times g$ for 60 minutes followed by further centrifugation of the resulting supernatant at $100,000\times g$ by Spinco L ultracentrifuge for 2 hours. For the actual test, viral pellets prepared after the ultracentrifugation from 1 ml, 3 ml and 5 ml of 20% infected brain suspension were mixed with 3 ml each of the hyperimmune sera diluted 1:10 in absorption tubes employing a sonicator. The mixtures were kept at 37°C for 30 minutes, then in a cold room for 16 hours. The sensitized mixtures were subjected for ultracentrifugation at $100,000\times g$ for 2 hours. The supernatants were heated at 56°C for 30 minutes to inactivate trace amount of infective virus and used for neutralization test as the absorbed sera.

Plaque reduction neutralization: Procedure for plaque production of JEV on chick embryo (CE) cell monolayers were essentially the same as described by Porterfield (1959). Briefly, chick embryo cell monolayers were prepared from 9 days old embryos. A sample from 2-fold serial dilutions of test sera was mixed with an equal volume of virus suspension containing 100 PFU in a final inoculum. The mixture was incubated in 37°C water bath for 1.5 hours, and inoculated to the monolayer cells. After 1.5 hours of adsorption, the monolayer was overlaid with the first overlay medium. The second overlay medium was applied 48 hours later. Plaques were counted the next day. Neutralizing antibody titers were expressed as reciprocals of the highest serum dilution showing reduction of plaque numbers more than 50% of that of control.

Hemagglutination (HA) assay: Preparation of sucrose-acetone (SA) antigens and measurement of HA activity with a microtiter was modified from the standard technique of Clarke and Casals (1958).

Purification of viruses: Viruses employed for purification were grown in BHK-21 monolayer cells as described elsewhere (Kitano, 1977). Viruses were purified by polyethylene glycol precipitation and two consecutive sucrose gradient centrifugation as described by Kitano (1977).

Polyacrylamide gel electrophoresis: Polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli (1970) in 12.5% acrylamide concentration. Samples were treated with 1.5% SDS and 1% 2-mercaptoethanol and heated at 100°C for 90 seconds. After electrophoresis, polypeptide bands were detected by staining using Coomassie Brilliant Blue.

RESULTS

Antibody-absorption test for antigenic analysis

Chinese A2 strain was compared with Japanese Nakayama-NIH and Mie 44-1 strains. Anti-A2 immune serum was absorbed by Nakayama-NIH or Mie 44-1 virus as well as by homologous A2 virus. The results are shown in Fig 1. Anti-A2 serum showed neutralizing antibody titer 1:640 when measured by three strains. Antibody titer dropped gradually down to 1:40 by increasing amount of absorbing homologous A2 virus 1.0 to 5.0. The same degree of absorption was observed when absorbed by Mie 44-1 strain. However, A2 specific antibody seemed not to be absorbed by Nakayama-NIH strain. Anti-Nakayama-NIH serum was thereupon absorbed with A2 virus. As shown in Fig 2, Nakayama-NIH specific antibody was consistently left even after increasing amount of heterologous absorbing virus. Such a difference of the absorption curve was observed more definitely when absorbed by Mie 44-1 virus. Neutralizing antibody titer 1:640 against Mie 44-1 virus in anti-Mie 44-1 serum was dropped remarkably when absorbed by A2 virus shown in Fig 3. However, Mie 44-1 specific antibody was clearly remained after absorption with Nakayama-NIH virus. Therefore, it seems reasonable to conclude that the antigenic composition of A2 virus is close to Mie 44-1 virus being definitely different from Nakayama-NIH virus.

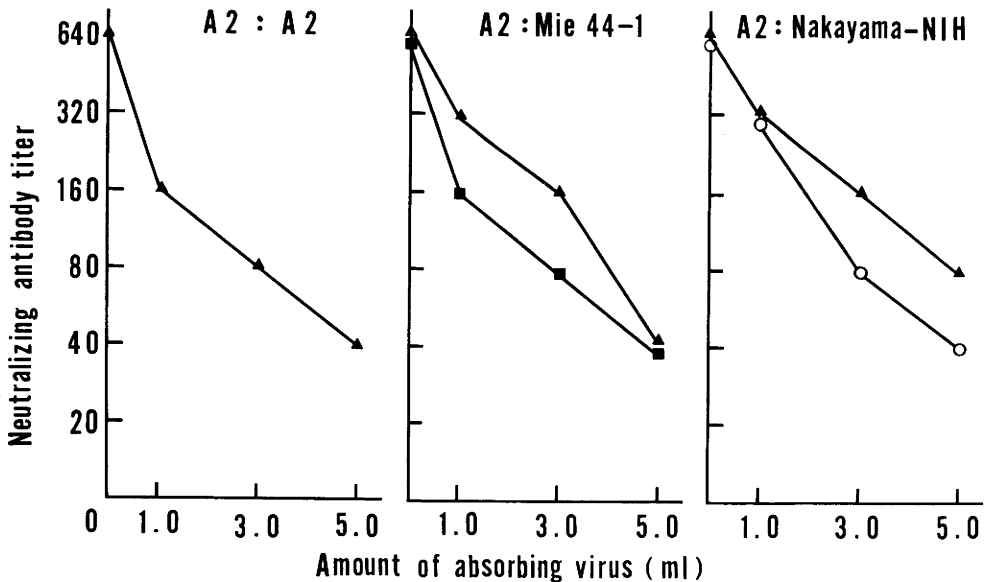


Fig 1. Absorption curve of anti-A2 antiserum by A2, Mie 44-1 or Nakayama-NIH virus. Anti-A2 antiserum was absorbed by various amounts of absorbing viruses, A2, Mie 44-1 and Nakayama-NIH. The residual neutralizing antibody titer was measured by A2 ▲—▲, Mie 44-1 ■—■, or Nakayama-NIH ○—○ virus.

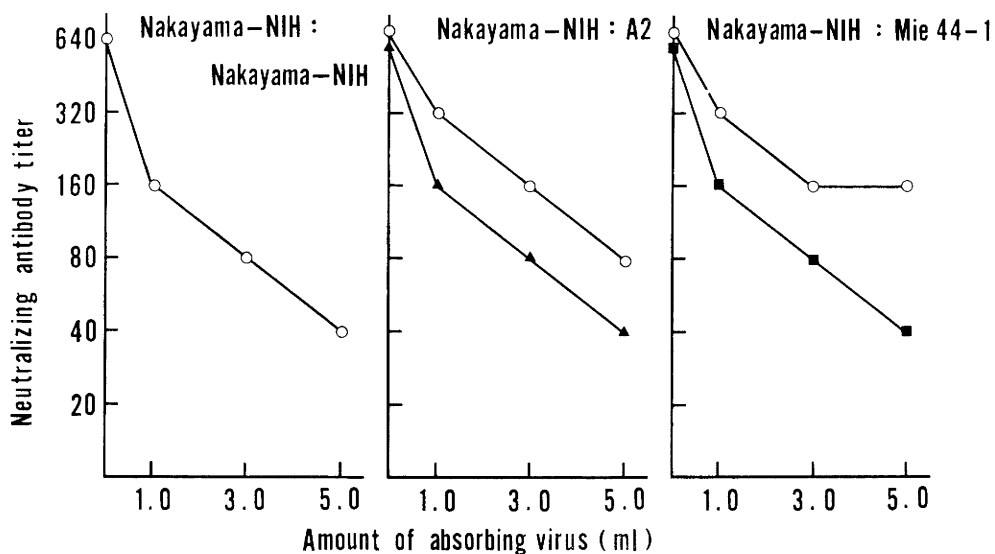


Fig 2. Absorption curve of anti-Nakayama-NIH antiserum absorbed by Nakayama-NIH, A2 or Mie 44-1 virus.
Anti-Nakayama-NIH antiserum was absorbed by various amounts of absorbing viruses, A2, Mie 44-1 and Nakayama-NIH. Symbols are the same as in Fig 1.

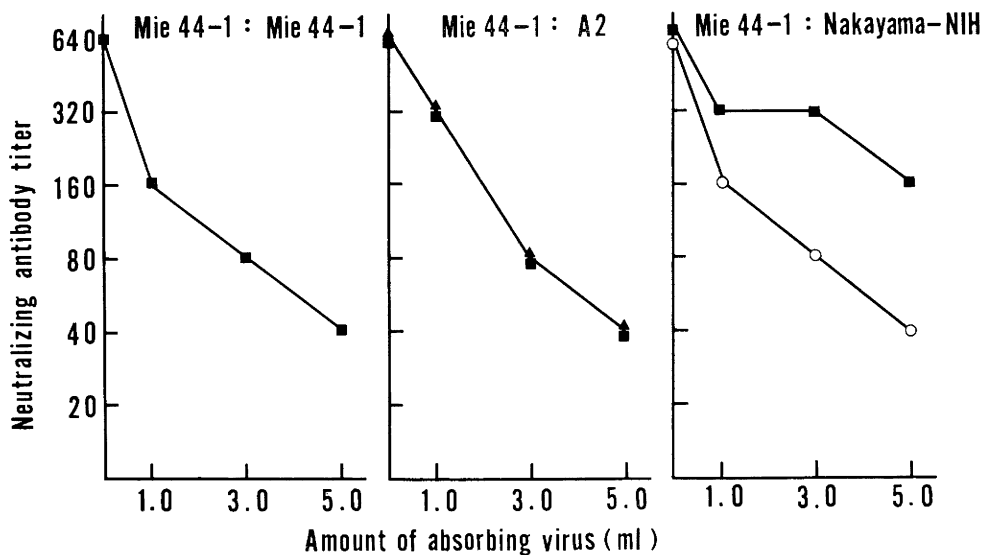


Fig 3. Absorption curve of anti-Mie 44-1 antiserum absorbed by Mie 44-1, A2 or Nakayama-NIH virus.
Anti-Mie 44-1 antiserum was absorbed by various amounts of absorbing viruses, A2, Mie 44-1 and Nakayama-NIH. Symbols are the same as in Fig 1.

pH-dependency and heat stability of HA

Since some immunological difference was observed in the surface antigens among three strains of JEV, the other properties of the surface proteins such as pH-dependency and heat stability of HA were examined. In addition to the above three strains, P3 and JaGAR-01 strains, each isolated in China and Japan, were employed in the experiment. Fig 4 shows the results of HA test at various pH with SA antigen prepared from suckling mouse brains infected with each strain. Strains Nakayama-NIH, A2 and P3 showed the similar HA pattern having the optimum pH at 6.4 or lower. On the other hand, the optimum pH of JaGAR-01 and Mie 44-1 strains were 6.8 or higher. Heat stability of those HA at their optimum pH were investigated at 37°C for the period of 150 minutes. As seen in Fig 5, HA of all strains except JaGAR-01 showed more or less stable characters at 37°C. However remarkable decrease in HA titer was observed in case of JaGAR-01 strain.

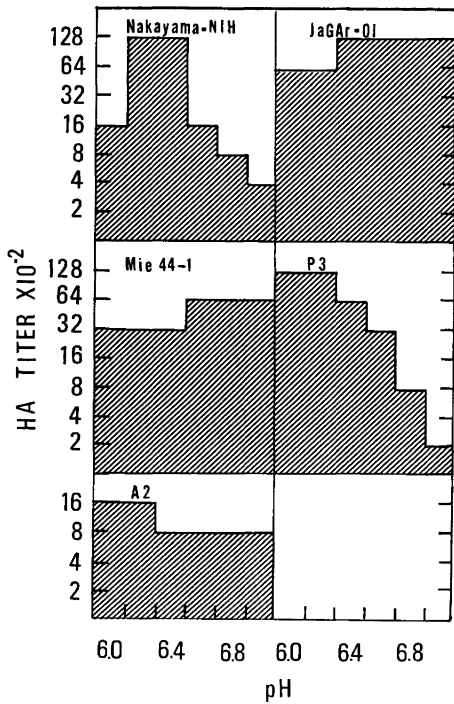


Fig 4. pH-dependency of hemagglutination of five JEV strains.

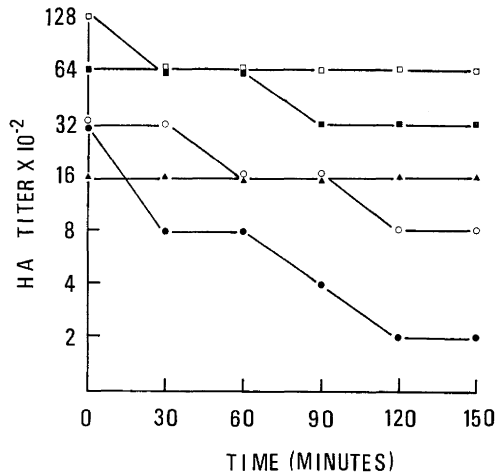


Fig 5. Heat stability of hemagglutinin of five JEV strains.

SA antigens dissolved in borate buffer pH 9.0 containing 0.4% bovine plasma albumin were incubated at 37°C.

At various time intervals, portion of samples were withdrawn and subjected for HA titration.

Note:

P3 □ — □, Mie 44-1 ■ — ■,
 A2 ▲ — ▲,
 Nakayama-NIH ○ — ○,
 JaGAR-01 ● — ●.

PAGE pattern of polypeptides from purified virions

A glycoprotein, one of the three structural polypeptides of JEV is known to show a hemagglutinating activity and to contain a strain specific antigen. Therefore, it was expected to show any molecular difference of glycoprotein among JEV strains by SDS-PAGE. Viral particles were purified from the infected culture fluids with A2, Nakayama-NIH and Mie 44-1 strains. The structural polypeptides were analyzed as described in Materials and Methods. Three species of structural polypeptides of JEV, envelope protein V1, V3 and core protein V2 were demonstrated from all strains. No difference in the mobility of these polypeptides was observed in PAGE with three strains employed in the experiment as shown in Photo 1.

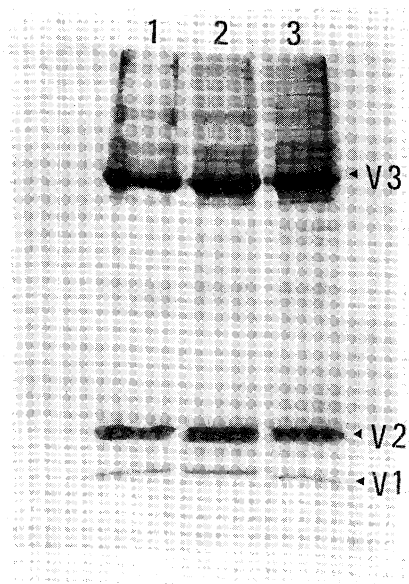


Photo 1. Comparison of structural polypeptides of three JEV strains by SDS-PAGE.

Note: 1 ——— Nakayama-NIH,
 2 ——— Mie 44-1,
 3 ——— A2.
 V1 ——— Membrane protein,
 V2 ——— Core protein,
 V3 ——— Glycoprotein.

DISCUSSION

Various methods have been applied to studies of the antigenic difference of JEV. Those were mouse protection test (Hale and Lee 1954), neutralization test (Kobayashi, 1959), kinetic neutralization test (Ozaki and Tabei, 1967), absorption test of neutralizing antibodies (Oda, 1976) and radioimmunoassay (Trent, 1977). The protection test is the method sensitive enough to detect minor antigenic difference. However, its results contained a wide error range which sometimes masked possible antigenic differences among JEV strains. Minor difference of antigenic structure of JEV was clearly demonstrated by antibody-absorption test or radioimmunoassay. Since our purpose of the present study is to know the epidemiological meaning of any antigenic difference between JEV strains isolated in China and Japan, antigenic difference of three JEV strains was examined by the antibody-absorption neutralization test. As seen in the text, some difference was observed between Chinese A2 strain and Japanese Nakayama-NIH strain, although A2 strain showed close antigenic similarity to a recent Japanese isolate, Mie 44-1 strain. Therefore, the observed antigenic difference between A2 and

Nakayama-NIH may reflect the difference of passage histories as suggested by Okuno et al. (1968).

It was reported that some properties of HA such as pH-dependency were changed during the passage in the laboratory (Okuno et al. 1965). Therefore, pH-dependency and heat stability of HA were examined on Chinese and Japanese strains. In our experiment, the optimum pH of A2 strain was lower, being different from Mie 44-1 and similar to Nakayama-NIH. The hemagglutinin of A2, Mie 44-1 and Nakayama-NIH were all stable at 37°C. Consequently, there seemed no relation between antigenic differences and pH-dependency or heat stability of JEV strains. JEV is known to have three structural polypeptides (Shapiro et al. 1971) and the major glycoprotein is essential for the expression of HA or neutralization (Kitano et al. 1974). By solid phase competition radioimmunoassay, Trent revealed that glycoproteins of flaviviruses also contain type-specific, complex-reactive and flavivirus group-reactive antigenic determinants (1977). However, no difference was observed by SDS-PAGE analysis in three structural polypeptides, including glycoprotein, of A2, Mie 44-1 and Nakayama-NIH strains. It is remained for further investigation in future to analyze the antigenicity of those polypeptides by more sensitive immunochemical method such as a radioimmunoassay as reported by Trent (1977).

At present, there is no definite evidence of geographical difference among strains of JEV isolated in various parts of the world. Therefore, it may be allowed reasonably to use a common JEV strain for diagnosis of JE or for vaccine production in any parts of endemic area of JEV in the Asian countries.

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日本と中国から分離された日本脳炎ウイルス株の免疫学，生物学と生化学性質の比較研究．

金 恩源（中国医学科学院．病毒学研究所），小林正美，緒方隆幸，北野忠彦，大谷 明（国立予防衛生研究所，ウイルス・リケッチャ部）

中国で分離された日本脳炎ウイルス（JEV）2株，A2株，P3株の免疫学的，生化学的性状を，日本で分離された3株，中山一予研株，JaGAR-01株，Mie 44-1株と比較する目的で抗体吸収試験，血球凝集（HA）試験，ポリアクリルアミド電気泳動（PAGE）を行なった．

その結果 A2株と中山一予研株の間には，共通抗原の他にそれぞれの株の特異抗原が存在することが認められた．一方Mie 44-1株と A2株では抗原性はほとんど一致した．A2株，P3株，中山一予研株の HA は至適 PH が6.4かそれ以下で，37°C の加熱に比較的安定であった．これに対して JaGAR-01 株は，至適 pH は6.8かそれ以上で，加熱に不安定であった．

このように，株間に免疫学的，生物学的に差がみられたのに対し，ウイルス蛋白の PAGE 上の挙動には株間の差はなかった．