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DIFFERENCES IN BIOLOGICAL ACTIVITY OF THE V3 ENVELOPE PROTEIN OF TWO JAPANESE ENCEPHALITIS VIRUS STRAINS

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Summary. — Two strains Nakayama-NIH and JaGAr-01 of Japanese encephalitis virus (JEV) distinctly differ in their virulence. Amino acid analysis of the V3 viral protein revealed different serine and tyrosine contents in JaGAr-01 V3 as compared to Nakayama-NIH V3. SDS-PAGE purified renatured JaGAr- and Nakayama-V3 proteins could absorb cross-reactive neutralizing antibodies present in anti-Nakayama and anti-JaGAr hyperimmune sera. They also absorbed strain specific antibodies of anti-JaGAr and anti-Nakayama sera, respectively. These observations clearly suggest that both JEV strain specific and cross reactive antigenic determinants are present on V3 protein molecules. Antibody against purified Nakayama V3 protein neutralized Nakayama-NIH virus, but had little neutralizing activity against JaGAr-01 virus. The phenomenon is less pronounced when antibodies against Nakayama-NIH virions are used rather than those against purified homologous V3. This suggests that the antigenicity of the purified V3 protein differs from that of the intact virus envelope.

Key words: Japanese encephalitis virus; envelope protein V3; strain difference

Introduction

Japanese encephalitis virus (JEV), belonging to genus *Flavivirus* of the family *Togaviridae*, is a small enveloped virus. It contains 3 structural proteins V1, V2 and V3, the latter being the envelope glycoprotein (Shapiro *et al.*, 1971, 1973). More than 50 JEV strains isolated in Japan since 1935, have been serologically classified into three subtypes: Nakayama-NIH, JaGAr-01 and Mie, the latter being an intermediate subtype between Nakayama-NIH and JaGAr-01. Nakayama-NIH (isolated in 1935) and JaGAr-01 (isolated in 1959) differ in their virulence (Oya *et al.*, 1961) and in their reactivity with the cell receptor for JEV (Yasui *et al.*, 1971). Moreover, the JaGAr-01 strain grows more rapidly in cultured cells and has a higher

Table 1. Molar percentage of amino acids in the JEV structural protein V3

Amino acid	Nakayama-NIH V3	JaGAr-01 V3
Lys	5.16	4.53
His	2.53	3.35
Arg	4.39	3.81
Asp	9.31	9.07
Thr	7.56	6.54
Ser	8.21	10.77
Glu	8.67	10.56
Pro	1.87	1.24
Gly	11.38	13.24
Ala	9.63	10.20
Cys	0.37	0.31
Val	8.66	8.39
Met	1.91	1.81
Ileu	4.43	3.71
Leu	8.94	7.06
Tyr	2.52	1.45
Phe	4.51	3.97

virulence in peripherally inoculated adult mice than the Nakayama-NIH strain. Finally, the Nakayama-NIH virus strain, inactivated with formalin, is a more effective vaccine than the inactivated JaGAr-01 strain (Oya, 1966).

Serological and biological differences between the two strains may be related to differences in their structural proteins, especially to those in the V3 glycoprotein. Here we report that biochemical and serological analyses of the highly purified and renatured V3 proteins from Nakayama-NIH and JaGAr-01 strains (Takegami *et al.*, 1982) showed remarkable differences in their composition and biological activity.

Table 2. Ability of the renatured JEV V3 protein to absorb the neutralizing antibody of anti-JEV serum

Antiserum	Antigen used for absorption	JEV strains used for the assay	Neutralizing titres of the antiserum						Activity
			Exp. I		Exp. II		Exp. III		
			C	T	C	T	C	T	
J	J V3	J	3.37*	2.80					+
J	J V3	N	2.99	2.99					−
J	N V3	J			3.48	3.60			−
J	N V3	N			3.07	2.00			+
N	J V3	J	3.08	2.59					+
N	J V3	N	3.62	3.56					−
N	N V3	J			2.64	2.18			+
N	N V3	N			2.99	2.93			−
N	N V3	N					3.15	3.15	−
N**	N V3	N					2.60	2.28	+

J = JaGAr-01, N = Nakayama-NIH, C = control, T = treated, N** = anti-Nakayama serum prepared by repeated adsorption to intact JaGAr-01 virions contained less cross-reactive neutrazing antibodies, *$\log_{10}$ values.

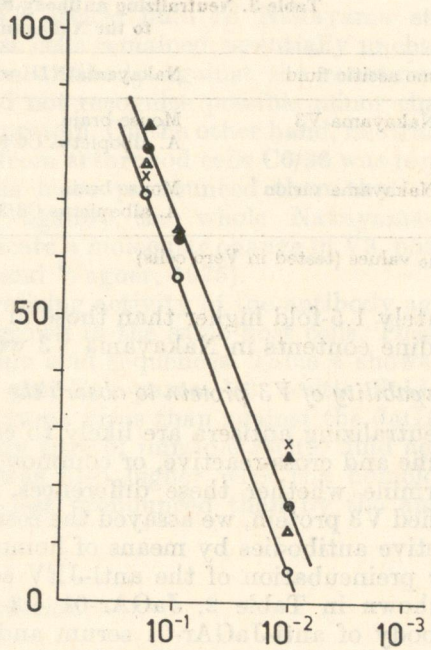

Fig. 1.

Neutralizing activity of the immune ascitic fluid prepared to the envelope protein V3 against JEV released from different hosts

Viruses were obtained from infected mouse brain (−△−) and the from culture media of various infected cells, such as Vero (− × −), LLC-MK2 (− ○ −), HEL (− ● −) and CEF (− ▲ −). Neutralizing activity was assayed in Vero cells. Abscissa: dilution of ascitic fluids; ordinate: plaque reduction (%).

Materials and Methods

Viruses and viral protein preparation. JEV strains Nakayama-NIH and JaGAr-01 were used. Purification and preparation of the envelope protein V3 were carried out as previously described (Takegami *et al.*, 1982).

Determination of amino acids. About 2 mg of purified lyophylized JEV structural protein was hydrolysed with 6 N HCl at 110 °C for 24 hr. The acid hydrolysate was analysed with an amino acid analyser (Hitachi KLA-5, Japan). Molar percentages for each amino acid were estimated using an authentic amino acid standard (Ajinomoto, Japan).

Assay of the ability of antigen to absorb neutralizing antibody. To examine the ability of V3 to absorb neutralizing antibody, anti-JEV hyperimmune sera were incubated with the renatured JEV V3 protein at 37 °C for 1 hr and the neutralizing antibody titers were measured by fifty per cent plaque reduction method using chick embryo fibroblasts (CEF).

Preparation of antibody against V3 protein. Mouse immune ascitic fluids against V3 protein were prepared as previously described (Takegami *et al.*, 1982). The neutralizing antibody titres were assayed by the fifty per cent plaque reduction method using CEF or Vero cells.

Results

Amino acid analysis

The method of protein V3 purification by SDS-PAGE (Takegami *et al.*, 1982) permitted to examine the amino acid composition of proteins isolated from different JEV strains. As seen in Table 1, glycine and alanine contents were high, whereas cystine and proline contents were low. A comparison of V3 proteins of the Nakayama-NIH and JaGAr-01 strains also revealed that the proline and tyrosine contents in Nakayama strains V3 were apro-

Table 3. Neutralizing antibody titres of immune ascitic fluide to the Nakayama-NIH strain

Immune ascitic fluid	Nakayama-NIH virus propagated in	Neutralizing titres
Anti-Nakayama V3	Mouse brain	1.70*
	A. albopictus C6/36 cells	0.85
Anti-Nakayama virion	Mouse brain	2.15
	A. albopictus C6/36 cells	1.80

* $\log_{10}$ values (tested in Vero cells)

ximately 1.5-fold higher than those in JaGAr-01 V3, whereas the serine and histidine contents in Nakayama V3 were lower than those in JaGAr-01 V3.

Capability of V3 protein to absorb the neutralizing antibody in anti-JEV sera

Neutralizing antisera are likely to contain mixtures of antibodies against specific and cross-reactive, or common, antigenic sites of the JEV virion. To determine whether these differences in antigenic sites reside also in the purified V3 protein, we assayed the residual titres of strain specific and cross--reactive antibodies by means of homologous and heterologous virus strains after preincubation of the anti-JEV serum with the renatured V3 protein. As shown in Table 2, JaGAr-01 V3 protein absorbed the strain specific antibody of anti-JaGAr-01 serum and the cross-reactive antibody of anti--Nakayama NIH serum (Exp. I). Alternatively, the Nakayama-NIH V3 protein also absorbed the cross-reactive antibody of anti-JaGAr serum. However, Nakayama-NIH V3 protein could not absorb the Nakayama strain specific antibody of the anti-Nakayama serum (Exp. II). This latter result is unexpected, since the Nakayama V3 protein could block the neutralizing activity to Nakayama virus in anti-Nakayama serum pretreated with JaGAr-01 virions (Exp. III).

Neutralizing activity of the antibody against V3 protein

Available evidence suggests that the envelope glycoproteins of togaviruses may change if the virus is grown in different host cells (Burke and Keegstra, 1976; Igarashi *et al.*, 1979). To examine the possible influence of the carbohydrate moiety in the virus neutralizing reaction, we allowed to react the mouse anti-V3 ascitic fluid with viruses originating from different hosts.

Table 4. Neutralizing antibody titres of immune ascitic fluids

Immune ascitic fluid	JEV strain used for assay	Neutralizing titres
Anti-Nakayama V3	Nakayama-NIH	2.52*
	JaGAr-01	0.8
Anti-Nakayama virion	Nakayama-NIH	2.75
	JaGAr-01	2.24

* $\log_{10}$ values (assayed in CEF)

Neutralizing activities of the anti-V3 ascitic fluid to Nakayama strains released from different vertebrate host cells remained essentially unchanged (Fig. 1). This fact indicatec that the antibody against the renatured V3 protein used in this experiment could not recognize possible minor changes in the carbohydrate moiety of the V3 protein. On the other hand, neutralizing activity to Nakayama-NIH released from arthropod cells C6/36 was remarkably decreased and the decrease was more pronounced than that in the neutralizing activity of antibodies against the whole Nakayama-NIH virions (Table 3). This result may indicate a molecular change in V3, possibly at the level of sialic acid (Schloemer and Wagner, 1975).

Next, we examined the virus neutralizing activity of the antibody against V3 protein of different JEV strains grown in the same host cells. These V3 proteins have probably different amino acid sequences. Table 4 shows that the neutralizing antibody titre of anti-Nakayama V3 ascitic fluid was significantly higher against the Nakayama virus than against the JaGAr-01 virus strain. The neutralizing antibody titre of immune ascitic fluid against the inactiveted Nakayama virions was also higher with the homologous virus strain, but the difference was less pronounced than by the anti-V3 ascitic fluid (Table 4).

Discussion

To elucidate the considerable differences in infectivity and pathogenicity of two JEV strains Nakayama-NIH and JaGAr-01, we examined the different properties of their structural proteins. Of the 3 structural proteins we concentrated our attention to protein V3, because it carries the bulk of the biological activities (Takegami *et al.*, 1982). Firstly, we examined the amino acid composition of the V3 protein and found it was rich in glycine and alanine, but relatively deficient in some basic amino acids (Table 1). Interestingly, V3 protein has an amino acid composition similar to the Sindbis envelope glycoproteins E1 and E2 (Burke and Keegstra, 1976) except for proline which is low in both the Nakayama-NIH and JaGAr-01 strains (Table 1). Our data indicate a difference between the serine, histidine, proline and tyrosine content of Nakayama-NIH and JaGAr-01 V3 proteins. This is clearly the result of the different genotypes of Nakayama-NIH and of JaGAr-01 viral strains. At present we are unable to correlate these differences with the biological functions of the envelope V3 protein.

The differences in structure of the V3 proteins are likely responsible for the differences in their antigenicity and reaction with antibodies (Tables 2—4). Remarkably, purified renatured V3 retained both cross-reactive and strain specific antigenic sites. In addition, V3 also appeared to carry flavivirus group-specific determinants (Della-Porta and Westaway, 1977; Trent, 1977). Based on our data we suggest that the virus envelope protein V3 may determine in major extent the biological activity of the two JEV strains.

The result that Nakayama-V3 protein could not absorb the neutralizing antibody to Nakayama-NIH virus from hyperimmune anti-Nakayama

serum but did absorb it from same serum pretreated with JaGAr-01 virions (Table 2) is difficult to explain. It could be due to a qualitative difference in neutralizing antibodies contained in anti-Nakayama and anti-JaGAr sera, and/or a difference in the affinity of cross-reactive antibody of anti-Nakayama serum to the common antigenic sites on V3 protein as compared to the affinity of strain specific antibodies and strain specific antigenic sites. Of interest is the finding that the strain specific antigenic sites of the Nakayama V3 protein were more effective in inducing the production of neutralizing antibody than the common antigenic sites on V3 (Table 4). It suggests that the antigenicity of the purified V3 protein differs from that of the intact virion.

In addition to protein structure, the antigenicity of the V3 protein is determined also by the carbohydrate moiety. This we conclude from experiments that the virus neutralizing titre of antibody against Nakayama V3 to the homologous Nakayama strain released from *A. albopictus* C6/36 cells was remarkably low in comparison with the titres to Nakayama strains released from various vertebrate cells (Fig. 1 and Table 3). Alternatively, JEV virions released from C6/36 cells may vary, and contain less V3 protein; such a phenomenon was observed with the G and L proteins of VSV (Gillies and Stollar, 1980). However it is difficult to imagine how carbohydrate influences the ability of antibody to adsorb to virions.

Finally, the different biological activity of viral strains may in some respect also be correlated to other viral proteins, such as V1. Shapiro *et al.* (1972, 1973) suggested that V1 protein is related to JEV infectivity and that it might be of taxonomic significance. Moreover, immune ascitic fluid against V1 protein had slight neutralizing activity to JEV (Takegami *et al.*, 1982). The biological function(s) of the V1 proteins of different viral strains are under investigation in this laboratory.

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