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BIOLOGICAL ACTIVITIES OF THE STRUCTURAL PROTEINS OF JAPANESE ENCEPHALITIS VIRUS

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Summary. — Structural proteins V1, V2 and V3 of Japanese encephalitis virus (JEV) were isolated and purified by means of polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) and extracted from the sliced gel. The purified and renatured V3 envelope protein was found to bind to receptors for JEV on red blood cells. V3 also bound to haemagglutination inhibition (HI) antibodies from anti-JEV sera. In addition, V3 protein induced neutralizing antibodies against JEV when injected into mice. These facts strongly suggest that V3 protein carries the antigenic epitope(s) that elicit neutralizing antibodies and react with HI antibodies. We conclude that V3 plays an important role in virus infection. V2 protein did not exhibit these biological activities, but antibody against V1 protein, not contaminated with V3 protein, showed slight virus neutralizing activity. Possible implications of these results are discussed.

Key words: Japanese encephalitis virus; structural proteins; biological activities

Introduction

Although both flaviviruses and alphaviruses have been classified as belonging to the family *Togaviridae*, recent reports indicate remarkable differences in virus RNA structure (Wengler *et al.*, 1978) and in the process of virus protein synthesis (Westaway, 1977). The mechanism of infection with and the biochemistry of multiplication of the Japanese encephalitis virus (JEV), member of the flavivirus genus, have been studied to some extent (Russell *et al.*, 1980; Westaway, 1980), but many questions remain unanswered. The cell receptor for JEV has been analyzed by Yasui and Nozima (1971) and Yasui *et al.* (1971), but the role of viral structural protein in the virus/host cell interaction is not well understood. JEV has 3 structural proteins: an envelope-bound glycoprotein (V3), an internal protein (V2) and a protein (V1) whose functions and precise location within the virion are not known. Previous studies have implicated that the envelope protein V3 of JEV has hemagglu-

tinin (HA) activity and also suggested that V3 is involved in the neutralizing reaction (Shapiro *et al.*, 1971; Kitano *et al.*, 1974). Insufficient removal of other proteins, however, left open the question whether V3 alone has these functions. This paper describes the complete separation of the envelope protein and correlates the purified and renatured polypeptides with their biological functions.

Materials and Methods

Virus. The strain Nakayama-NIH of JEV, propagated in mouse brains, was used in all experiments.

Virus purification. JEV-infected mouse brains were homogenized in phosphate buffered saline (PBS) (pH 7.6) and centrifuged at 10,000 × g with a continuous flow rotor. Ethyl alcohol (final concentration 20%) was added to the supernatant. The mixture was kept overnight at 8 °C and centrifuged. The resultant precipitate was resuspended in PBS and clarified by centrifugation. Protamine sulfate (0.2 mg/ml) was added to the supernatant and the mixture was again centrifuged. The final supernatant was applied on a 0—80% (w/v) sucrose density gradient and continuous rate zonal centrifugation was performed at 53,000 rev/min with a RK type J-1 rotor (Electro Nucleonica Inc., U.S.A). After fractionation, the HA peak fraction was concentrated by ultracentrifugation at 105,000 × g for 2 hr.

Preparation of ^{3}H-labeled JEV

Cultured chick embryo fibroblast (CEF) were infected with JEV at a multiplicity of infection (m.o.i.) of 0.01 PFU/cell. After incubation for 1 day in Eagle's minimum essential medium (MEM) lacking serum at 37 °C, a mixture of [^{3}H] amino acids (Radiochemical Center, Amersham) was added to the medium (final concentration of 130 kBq/ml) and the cells were incubated further for 2 days. To purify the JEV particles, the culture medium was centrifuged at 10,000 × g for 30 min and the supernatant was layered on a discontinuous gradient of 10% sucrose in 0.05 mol/l Tris-HCl (pH 8.0) buffer containing 0.14 mol/l NaCl and 0.1 mg/ml cystine, and 30% sucrose plus 25% metrizoate mixture in Tris buffer. The sample was centrifuged at 24 000 rev/min for 2 hr with a swinging rotor (Hitachi RPS 25). The peak fraction of HA activity was collected and centrifuged at 105,000 × g for 90 min. The resulting pellet was used as ^{3}H-labeled JEV.

Purification of JEV structural protein (SDS-gel slicing method). Disc gels containing 0.1% SDS were polymerized at 10% acrylamide, 0.3% bisacrylamide and electrophoresed at room temperature. After preincubation with 1% SDS and 1% 2-mercaptoethanol (ME) at 37 °C for 30 min, purified JEV was layered on the top of a gel and electrophoresed at 3 mA for the first 10 min, and then at 5 mA until the dye front had migrated approximately 6 cm. After the run, one of the gels was stained with 0,1% Coomasie brilliant blue in 10% acetic acid and 25% methylalcohol. The other gels were sliced at sites where the JEV structural proteins were situated by the stain. The slices were homogenized in a Teflon homogenizer with 0.02 mol/l phosphate buffer (pH 7.2), centrifuged at 10,000 × g for 20 min and the supernatant was dialysed against phosphate buffer (pH 7.2) for 24 to 48 hr.

Renaturation of JEV structural proteins. SDS was removed from dissociated JEV structural protein by ion exchange column chromatography according to the method of Weber and Kuter (1971). Briefly, JEV protein was mixed with mouse serum albumin (2 mg/ml) and dialyzed against 0.05 mol/l Tris-HCl buffer (pH 7.5) containing 6 mol/l urea, 0.1% 2-ME, 0.1 mmol/l EDTA at room temperature for 4 hr. The sample was layered onto a Dowex AG 1 × 2 column (5 × 30 mm). The protein fraction eluted with elution buffer [6 mol/l urea, 0.1% 2-ME, 0.1 mmol/l EDTA and 0.05 mol/l Tris-HCl buffer (pH 7.5)] was collected and added drop by drop into 10 volumes of 0.05 mol/l Tris-HCl buffer (pH 7.5) containing 0.1% 2-ME and 0.1 mmol/l EDTA. The protein was then concentrated by passing it through a PM-10 ultrafilter and then dialysed against Tris-HCl buffer (pH 7.5) containing 0.1 mmol/l EDTA at 4 °C. The dialysed protein fraction was used as renatured JEV structural protein.

Sucrose gradient analysis of renatured ^{3}H-V3 protein. ^{3}H-labeled V3 protein, from which SDS had been removed by ion exchange chromatography, was layered on a 15—30% sucrose gradient in Tris-HCl buffer (pH 7.5) containing 0.14 mol/l NaCl and 1 mmol/l EDTA, and centrifuged

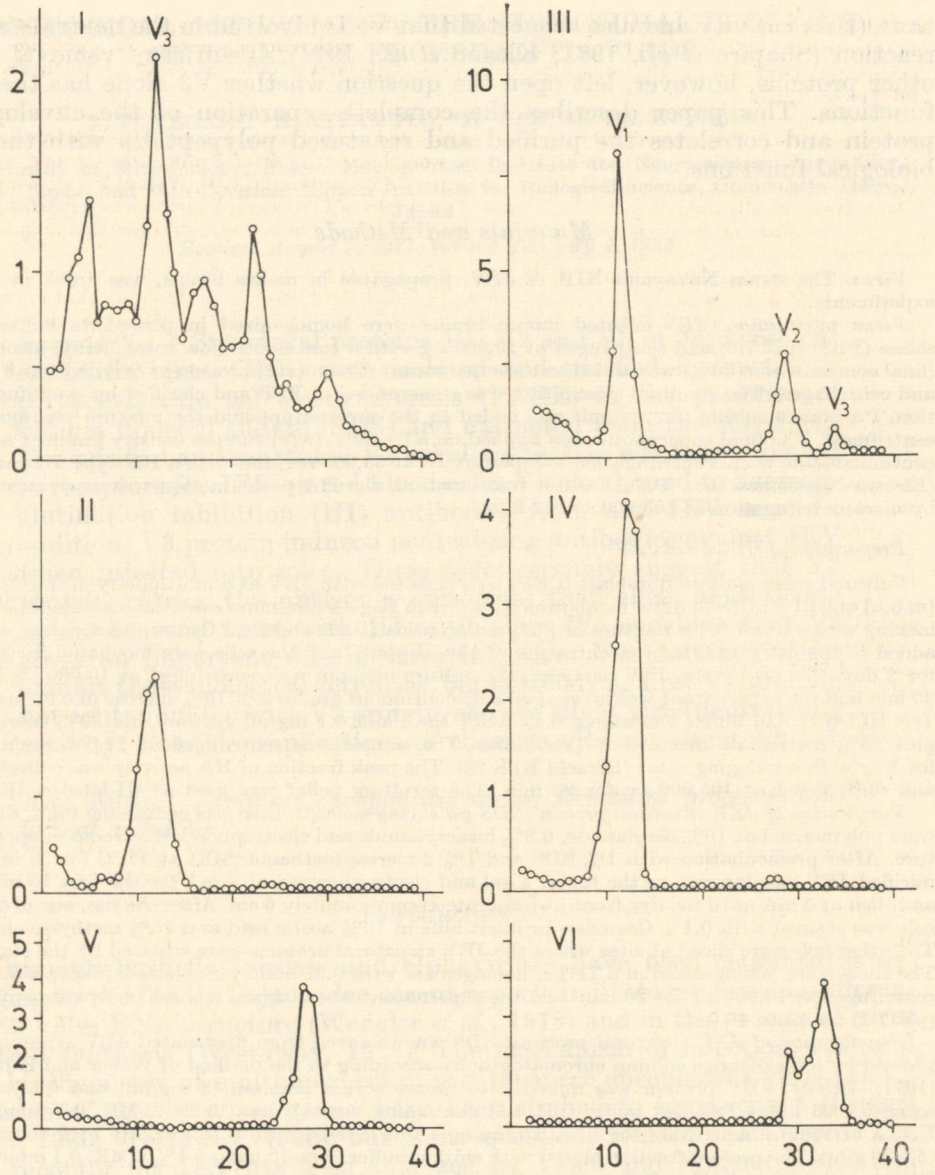

Fig. 2.

Immunoprecipitation with antisera against JEV structural proteins

Radioimmunoprecipitation was carried out as described in Materials and Methods. Briefly, JEV-infected cells or JEV virions were incubated with antiserum against JEV structural protein at 37 °C for 1 hr and overnight at 4 °C. After addition of antiserum against rabbit or mouse IgG, the mixture was centrifuged.

at 24,000 rev/min for 15 hr with a Hitachi 25 RPS rotor. The fractions were monitored spectrophotometrically at 280 nm and radioactivity was counted with a liquid scintillation spectrometer.

Immunodiffusion test. The immunodiffsion test was carried out according to Ouchterlony and Nilson (1973).

Preparation of rabbit or mouse antisera and mouse ascitic fluids containing antibodies against virus components. Adult male rabbits were inoculated with Freund's complete adjuvant (containing approximately 1 mg purified V3. Sera were harvested after 3 injections at one week intervals. About 50 μg of each of the three renatured JEV proteins (i.e. V_1, V_2 and V_3) were injected intraperitoneally into adult BALB/c mice. Two weeks later, a second injection was performed and on the following day, ascitic cells were injected. Mouse immune sera and ascitic fluids were harvested 3 weeks after the first injection.

Indirect immunofluorescence test. JEV-infected LLC-MK2 cells were fixed in acerone and incubated with antisera against each JEV component at 37 °C for 1 hr. After incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG, the cells were viewed in fluorescence microscope.

Radioimmunoprecipitation. 100 μl of ^{3}H-labeled JEV virion or ^{3}H-labeled cell extract solubilized in Tris-HCl buffer (pH 7.5) containing 0.14 mol/l NaCl, 2% NP-40, 1% Triton X-100 and 0.05% SDS, was incubated with 20 μl of rabbit or mouse antisera agains JEV structural protein for 1 hr at 37 °C and overnight at 4 °C. After addition of anti-rabit or anti-mouse IgG, the mixtue was incubated at 37 °C for 1 hr and centrifuged at 5,000 × g for 10 min. The immunoprecipitate was washed four times in Tris buffer containing 0.14 mol/l NaCl, 1% NP-40 and 0.05% SDS. The washed pellet was resuspended in electrophoresis buffer containing 1% SDS and 1% 2-ME and analysed by SDS-PAGE.

Assay of neutralizing antibody titers to JEV. Fifty per cent plaque reduction method in CEF or Vero cells was used to assay the neutralizing antibody titers of sera and ascitic fluids. In the Vero cell system plaque assay was carried out by a new method using anti-JEV sera and complement (Furukawa and Yasui, 1978).

Ability of the proteins to absorb hemagglutination inhibition (HI) antibodies. After antisera against JEV were incubated at 4 °C overnight with renatured JEV proteins, remaining HI antibody titers were measured by microtiter technique using pigeon red blood cells (Mori *et al.*, 1965).

Results

Isolation of JEV structural proteins

Fig. 1 shows the SDS-PAGE pattern of the separation of V1, V2 and V3 proteins. Each protein was completely dissociated from the other ones. We found that SDS-PAGE is superior to other dissociation procedures using gel filtration or isoelectric focusing (data not shown). The estimated molecular weights of structural proteins were 53,000 (V3), 14,000 (V2) and 9,500 (V1); these data are similar to those reported by Shapiro *et al.* (1971) and Westaway (1973).

Radioimmunoprecipitation clearly indicated that anti V3 rabbit serum could specifically bind to V3 protein in SDS-disrupted JEV preparation, and also to intracellular V3 protein in JEV infected cells (Fig. 2-II and 2-IV). Figs 3 and 4 show JEV infected cells analysed by indirect immunofluorescence. Bright fluorescence was observed in the perinuclear cytoplasm of JEV infected LLC-MK2 cells stained with anti-V3 serum (Fig. 4).

Figures 2-I and 2-II show the SDS-PAGE pattern of ^{3}H-labeled polypeptides in JEV infected cell extract and immunoprecipitable polypeptides with anti-V3 rabbit serum from infected cell extract, respectively. Fig. 2-III shows SDS-PAGE pattern of JEV structural proteins. Further figures indicate JEV structural proteins immunoprecipitated with rabbit antiserum against V3 (2-IV) and mouse antiserum against V2 (2-V) or V1 (2-VI), respectively.
Abscissa: slice number; ordinate: ^{3}H-radioactivity (cpm × 10^{-4})

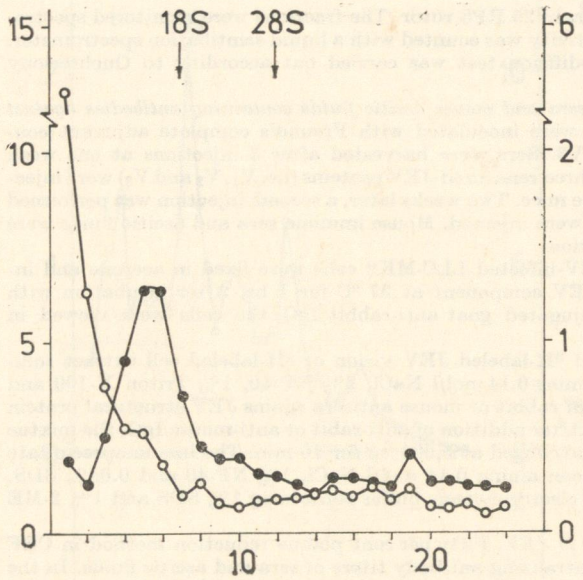

Fig. 5.

Sucrose gradient analysis of the renatured ^{3}H-V3 protein

^{3}H-V3 protein renatured by ion exchange column chromatography was layered on a 15—20% sucrose gradient and centrifuged at 14,000 rev/min for 15 hr. Arrows indicate the position of cellular rRNA added as marker.

Denatured V3 protein (—○—); renatured V3 protein (—●—).

Abscissa: fraction number; 1st ordinate: ^{3}H-radioactivity (cpm$\times 10^{-2}$); 2nd ordinate: cpm$\times 10^{-3}$.

Reactivation of SDS-denatured proteins

SDS-PAGE seems very useful in isolation of JEV proteins. However, the method may cause a loss in the HA activity of isolated envelope protein and may destroy antigenic determinants present in the native polypeptide only. Indeed, rabbit antiserum against SDS-denatured V3 protein did neither neutralize virus infectivity nor inhibit hemagglutination (data not shown). To determine the biological activity of JEV structural protein, we attempted to remove SDS from purified protein fraction by ion exchange column chromatography. In this experiment, mouse serum albumin was added to retard nonspecific adsorption of JEV protein to the column matrix. The renatured V3 protein appears to form an aggregate sedimenting at 15 S in the absence

Table 1. The inhibition of hemagglutination (HA) reaction by pretreatment of red blood cells with renatured V3 protein

Amounts of renatured V3	Inhibition of HA
0	—
1*	±
2	+
4	++
7	+++

Pigeon red blood cells were mixed with the renatured V3 in Falcon Micro Test 113042 plate and incubated at room temperature for 60 min. After addition of 4 HA units of virus (Nakayama strain), the mixture was incubated at 35 °C for 30 min. HA was observed by inverted microscopy.

* Values show relative amounts of the renatured V3 protein in reaction medium before treatment with pigeon red blood cells.

Table 2. Capability of renatured JEV proteins to absorb HI antibody of the anti-JEV serum

Experiment	Antigen	HI antibody titers
I	Buffer	128
	Renatured V3 protein	16
II	Buffer	32
	Renatured V2 protein	32
	HA antigen*: 60 HA units	16
	3200 HA units	< 2

* The HA antigen was prepared from JEV-infected mouse brain by sucrose-acetone extraction.

of SDS (Fig. 5). Addition of SDS to the aggregate dissociates it and a single peak of V3 protein was obserwed (data not shown). The recovered proteins free of SDS were used as renatured JEV proteins in the following experiments.

Biological activities of the renatured JEV proteins

Significant HA activity of the renatured V3 was not detected. However, the fact that HA activity of JEV virions was inhibited when red blood cells were pretreated with renatured V3 (Table 1) suggests an interaction between the renatured V3 and red blood cells receptor for JEV. A precipitation line of

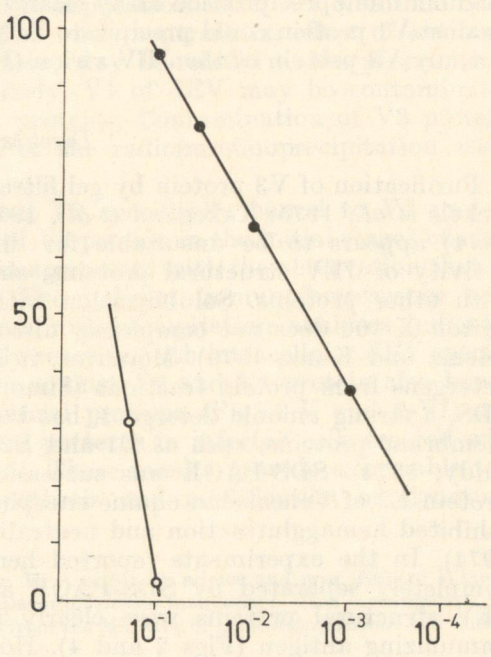

Fig. 6.
Neutralizing activity of mouse antisera against the renatured JEV proteins ■
Titration of neutralizing antibody ▼ was carried out in the Vero assay system; anti-V-3 serum (—●—); anti-V1 serum (—○—).
Abscissa: serum dilution; ordinate: plaque reduction (%)

Table 3. Neutralizing antibody titers of immune ascitic fluids*

Immune ascitic fluid	Neutralizing antibody titer
Anti-V3	2.52
Anti-V2	0.8
Anti-V1	1.43

* Immune ascitic fluids against renatured Nakayama-NIH structural proteins.
** Neutralizing activity to Nakayama-NIH virus was tested in CEF ($\log_{10}$ values).

the renatured V3 with anti-JEV serum was also observed in the immunodiffusion test (data not shown). Table 2 shows that renatured V3 protein adsorbed the HI antibody of anti-JEV serum. On the other hand, renatured V2 protein could not adsorb the HI antibodies to JEV.

Neutralizing activity in anti-renatured JEV protein sera and immune ascitic fluids

Although rabbit antiserum against SDS-denatured V3 protein showed no neutralizing antibody activity, mouse antiserum and immune ascitic fluids against the renatured V3 indicated a high neutralizing activity to Nakayama virus strain (Table 3 and Fig. 6) and reacted with V3 protein by immunoprecipitation (data not shown). Interestingly, we could detect slight neutralizing activity to JEV in immune ascitic fluid and in serum raised against the renatured V1 protein, but not in those formed against V2 protein (Table 3). Radioimmunoprecipitation assay clearly indicated that the mouse antiserum against V1 protein could precipitate V1 protein and a part of V2 protein but not any V3 protein of the JEV virion (Fig. 2-VI).

Discussion

Purification of V3 protein by gel filtration (Simons and Kääriäinen, 1970; Eckels *et al.*, 1975; Takegami *et al.*, 1977) and cetrifugation (Kitano *et al.* 1974) appears to be unsuitable for direct examination of the biological activity of JEV structural proteins, since the V3 remained contaminated with other proteins. Solubilization with non-ionic detergents, NP-40 and Triton X-100 does not completely dissociate V3 and V1 envelope proteins (Heinz and Kunz, 1979). Moreover, it is difficult to remove the non-ionic detergens from protein fractions (Simons *et al.*, 1973). On the other hand, SDS, a strong anionic detergent, has been found useful for the isolation of membrane proteins, such as E1 and E2 alphavirus proteins (Pederson and Eddy, 1974). SDS-PAGE was successfully used to isolate the membrane protein E2 of Venezuelan equine encephalitis virus and antiserum against it inhibited hemagglutination and neutralized the virus (Pederson and Eddy, 1974). In the experiments reported here, 3 JEV structural proteins were completely separated by SDS-PAGE and antisera against the individual JEV structural proteins were clearly monospecific with respect to their immunizing antigen (Figs 2 and 4). However, V3 purified in the presence

of SDS did not bind to red blood cells, and antiserum directed against it had no HI or neutralizing activity. Eckels *et al.* (1975) had already reported that in the presence of SDS, complement fixation activity of JEV was retarded while the HA and infectivity were decreased. These results are probably due to protein denaturation by SDS (Tanford, 1970). For analyses of some biological activities of JEV structural proteins, it is necessary to remove SDS from the SDS-protein complex allowing the protein to regain its original structure. Therefore we used ion exchange chromatography to separate SDS from protein. This procedure was also used for the purification of retrovirus glycoproteins (Schetters and McLeod, 1979). Renatured V3 of JEV indeed had an affinity to the receptor for JEV on red blood cells (Table 1) (Yasui *et al.*, 1971). In addition, renatured V3 protein exhibited remarkable activities absorbing HI and neutralizing antibodies (Table 2; Takegami *et al.*, 1982) and producing neutralizing antibody to JEV in mice (Table 3 and Fig. 6). These results strongly suggest that V3 protein plays a vital role in virus infection, a conclusion consistent with a previously published report (Kitano *et al.*, 1974).

Interestingly, antibody against V1 protein possessed slight virus neutraizing activity (Table 3 and Fig. 6). The role of V1 protein in flavivirus infection and replication or in viral architecture is unknown (Russell *et al.*, 1980 Westaway, 1980). If the result shown in Table 3 and Fig. 5 is true, it is likely that a part of the V1 protein is present on the virion surface. Recently, Lee and Schloemer (1981) reported that V1 isolated from Banzivirus, another flavivirus, interfered with Banzivirus replication when V1 was incubated with host cells prior to infection. The virus neutralizing effect of antibody against JEV-V1 protein, reported here, may be related to the phenomenon described for Banzivirus. Alternatively, V1 of JEV may be contaminated with small amounts of other JEV proteins. Contamination of V3 protein, however, is very unlikely because of the radioimmunoprecipitation assay shown in Fig. 2-VI.

Although serum against renatured V2 specifically bound to V2 protein (Fig. 2-V), any biological activities of V2 protein in the initial stages of virus infection were not detected. This is in agreement with the observation that V2 is an internal structural protein. The indirect immunofluorescence with FITC-labeled antibodies and radioimmunoprecipitation analyses indicated that rabbit anti-V3 serum was highly reactive with intracellular JEV specific protein V3 (Figs 2 and 3). Antisera against V2 and V1 protein also bound specifically to the respective structural proteins (Fig. 2-V and 2-VI) and revealed specific immunofluorescence patterns in infected cells treated with corresponding sera (data not shown). These recults provide a valuable tool for the examination of the biosynthesis and modification of structural proteins in JEV-infected cells.

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References

Eckels, K. H., Hetrick, F. M., and Russell, P. K. (1975): Virion and soluble antigens of Japanese encephalitis virus. *Infect. Immun.* **11**, 1053—1060.

Furukawa, J. K., and Yasui, K. (1978): New plaque method for JEV titration by use of anti-JEV serum and complement (in Japanese). The 26th Meeting of Society Japan Virologists, Tokyo.

Heinz, F., and Kunz, C. (1979): Dissociation of tick-borne encephalitis virus by Triton X-100 and acetyltrimethylammonium bromide. *Acta virol.* **23**, 189—197.

Kitano, T., Suzuki, K., and Yamaguchi, T. (1974): Morphological, chemical, and biological characteriza+ion of Japanese encephalitis virus and its hemagglutinin. *J. Virol.* **14**, 631—639.

Lee, C., and Schloemer, R. H. (1981): Identification of the antiviral factor in culture medium of mosquito cells persistently infected with Banzi virus. *Virology* **110**, 445—454.

Mori, H., Nakai, H., and Nozima, T. (1965): The interaction of Japanese encephalitis virus with its receptor prepared from pigeon red blood cells and susceptible cells. *Acta virol.* **9**, 97—108.

Ouchterlony, O., and Nilson, L. A. (1973): Immunodiffusion and immunoelectrophoresis, p. 39. In D. W. Weir (Ed.): *Handbook of Experimental Immunology*, Blackwell Scientific Publishers, Oxford.

Pedersen, C. E., and Eddy, G. A. (1974): Separation, isolation and immunological studies of the structural proteins of Venezuelan equine encephalitis virus. *J. Virol.* **14**, 740—744.

Russell, P. K., Brandt, W. E., and Dalrymple, J. M. (1980): Chemical and antigenic structure of flaviviruses, pp. 503—529. In. R. W. Shlesinger (Ed.): *The Togaviruses*, Academic Press, New York.

Schetters, H., and McLeod, B. (1979): Simultaneous isolation of major viral proteins in one step. *Anal. Biochem.* **98**, 329—334.

Shapiro, D., Brandt, W. E., Cardiff, R. D., and Russell, P. K. (1971): The proteins of Japanese encephalitis virus. *Virology* **44**, 108—124.

Simons, K., and Kääriäinen, L. (1970): Characterization of Semliki forest virus core and envelope protein. *Biochem. biophys. Res. Commun.* **38**, 981—988.

Simons, K , Helenius, A., and Garoff, H. (1973): Solubilization of the membrane proteins from Semliki forest virus with Triton X-100. *J. mol. Biol.* **80**, 119—133.

Takegami, T., Miyamoto, H., Nakamura, H., and Yasui, K. (1977): Isolation and characterization of Japanese encephalitis virus structural proteins (in Japanese). The 50th Meeting of Japan Biochemist Society, Tokyo.

Takegami, T., Miyamoto, H , Nakamura, H , and Yasui, K. (1982): Differences in the biological activity of the V3 envelope protein of two Japanese encephalitis virus strains. *Acta virol.* **26**, 321—327.

Tanford, C. (1970): Protein denaturation. *Advance in Protein Chem.* **24**, 1—95.

Weber, K., and Kuter, D. J. (1971): Reversible denaturation of enzymes by sodium dodecyl sulfate. *J. biol. Chem.* **246**, 4504—4509.

Wengler, G., Wengler, G. and Gross, H. J. (1978): Studies on virus-specific acid synthesized in vertebrate and mosquito cells infected with flaviviruses. *Virology* **89**, 423—437.

Westaway, E. G. (1973): Protein specified by group B togavirus in mammalian cells during productive infections. *Virology* **51**, 454—465.

Westaway, E. G. (1977): Strategy of the flavivirus genome: Evidence for multiple internal initiation of translation of proteins specified by Kunjin virus in mammalian cells. *Virology* **80**, 320—335.

Westaway, E. G. (1980): Replication of flaviviruses, pp. 531—581. In R. W. Schlesinger (Ed.): *The Togaviruses*, Academic Press, New York.

Yasui, K., and Nozima, T. (1971): Partial in vitro reconstruction of Japanese encephalitis virus receptor. *Acta virol.* **15**, 113—118.

Yasui, K., Nozima, T., Homma, R., and Ueda, S. (1971): Extraction of active fragment from Japanese encephalitis viral receptor of susceptible cells. *Acta virol.* **15**, 7—18.

Explanation of Micrographs (Plate XXXIX):

Indirect immunofluorescence staining of JEV infected LLC-MK2 cells. LLC-MK2 cells infected with JEV were incubated for 34 hr and fixed with acetone. Thereafter, cells were incubated at 37 °C for 1 hr with rabbit anti-JEV serum (Fig. 3) or with rabbit anti-V3 serum (Fig. 4). After washing with PBS, cells were incubated with FITC-conjugated anti-IgG.

Fig. 3. — Specific fluorescence with anti-JEV serum.

Fig. 4. — Specific fluorescence with anti-V3 serum.