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Recovery of Japanese Encephalitis Virus Protein after Phenol Extraction of the Purified Virus

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Abstract: Envelope glycoprotein of Japanese encephalitis (JE) virus was recovered from the mixture of the phenol and interphase after phenol extraction of the purified virus to obtain viral RNA from aqueous phase. The protein showed the same electrophoretic mobility as the V3 protein by the polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) and was shown to retain at least some of its antigenicities by the Western blotting using polyclonal anti-JE virus rabbit serum. The fine structure of the recovered protein should be examined for its antigenicity, immunogenicity as well as molecular integrity.

INTRODUCTION

Since its first application to obtain RNA from tobacco mosaic virus by Gierer and Schramm (1956), phenol extraction has extensively been used as the standard method to obtain various high molecular weight RNAs with intact biological activities. In the case of JE virus, which belongs to Flaviviridae, phenol extraction of the virus or the virus-infected tissues, could give RNA preparation possessing the infectivity of the virus (Nakamura, 1961; Igarashi *et al.*, 1963). In this laboratory, studies have been made to analyze genome RNA of JE virus, in order to elucidate various biological and epidemiological aspects of the virus, for example, RNA fingerprinting to show the strain differences (Hori *et al.*, 1986), or to analyze base sequence of viral genome in order to utilize the informations to develop the second generation vaccine. During these studies, quantities of the purified virus preparations were extracted with phenol in order to prepare viral RNA. However, little attention has been paid to the possibility that the viral protein could be present in the phenol or the interphase. We tried to see whether the viral protein could be recovered from the residual mixture of the phenol and the interphase after viral RNA

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was obtained as the aqueous phase. The present paper describes successful recovery of viral envelope glycoprotein, V3 (E), which still retained at least some of its antigenicities as shown by the Western blotting.

MATERIALS AND METHODS

Cells: *Aedes albopictus*, clone C6/36, cells (Igarashi, 1978) were grown at 28°C as mass culture in 1 liter of suspension in a spinner bottle using 2 mg/ml of microcarrier (Cytodex I, Pharmacia, Sweden) with cell growth medium which consists of Eagle's minimal essential medium supplemented with 10 % heat-inactivated fetal calf serum and 0.2 mM each of nonessential amino acids (Eagle, 1959).

Virus: A wild strain of Japanese encephalitis (JE) virus, JaOArS982, which was isolated from *Culex tritaeniorhynchus* in Osaka Prefecture in 1982, was kindly supplied by Dr. Ueba of the Osaka Prefectural Institute of Public Health and was used in the experiment.

Virus inoculation, purification and phenol extraction: Growth medium was removed from the C6/36 cell culture by decantation and 20 ml of the seed virus was inoculated. Virus adsorption was proceeded at room temperature for 2 hours and 750 ml of the maintenance medium (cell growth medium from which serum concentration was reduced to 2 %) was added and the cells were incubated at 28°C. Infected culture fluid was harvested every 24 hours and the cells were fed with fresh maintenance medium for altogether 4 days. The infected culture fluid was pooled (total volume 3 liters) and centrifuged at 3,000 rpm for 15 min. The supernatant was added with polyethylene glycol 6000 and NaCl to 6 % and 0.5 M final concentrations, respectively. After polyethylene glycol was completely dissolved, the mixture was centrifuged at 10,000×g for 30 min. The supernatant was removed and the precipitate was resuspended in 15 ml of STE buffer (0.1 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA, pH 7.4) and centrifuged at 10,000×g for 15 min. The supernatant was loaded onto 10 ml of 30-50 % sucrose gradient and 3 ml of 15 % sucrose in STE buffer and was centrifuged at 22,000 rpm for 16 hours at 4°C in an SW25.1 rotor of a Beckman model L5-50 ultracentrifuge. Fractions of 0.6 ml volume were collected by an ISCO density gradient fractionator model 640 and peak fractions of OD_{254} were pooled as purified virus fractions. The fractions were diluted 1:4 with STE buffer and the virus was pelleted by centrifugation at 40,000 rpm for 90 min in an SW 50.1 rotor. The pellet was resuspended in 0.5 ml of STE buffer by brief freezing-thawing and SDS was added to final 0.1 %. Immediately, RNA was extracted with the same volume of phenol saturated with STE buffer at room temperature for 5 min and the phase was separated by centrifugation at 10,000×g for 5 min. The aqueous phase was again extracted with the same volume of STE-saturated phenol and the aqueous phase was used as viral RNA preparation.

Recovery of viral protein from combined phenol and interphase: The residual phenol and interphase of 2 extraction steps were pooled and phenol was removed by repeated extraction with ethylether. Since the phenol and interphase contained small amount of water, the viral proteins which remained in the phenol and interphase were concentrated in the remaining water phase or sometimes become amorphous precipitate. The protein was then precipitated by adding acetone with 20 times or more volume of the remaining water and was collected as pellet by centrifugation at 10,000×g for 15 min and was vacuum dried.

SDS-PAGE: Slab gel method (Studier, 1973) with discontinuous buffer system (Laemmli, 1970) was used with 10 % separation gel (acrylamide: bisacrylamide ratio of 30: 0.8) in 1mm thinkness. Protein precipitate was tried to solubilize with 0.125 M Tris-HCl, pH 6.8, containing 2 % SDS and 5 % 2-mercaptoethanol at 100°C 1 min, however, often massive precipitate was hard to become uniform solution. The solubilized supernatant was separated by the SDS-PAGE and the part of the gel was stained with 0.1 % Coomassie Brilliant Blue, R250, in 10 % acetic acid and 30 % methanol followed by destaining with 10 % acetic acid and 30 % methanol. The remaining part of the gel was used for electro-transfer or electroelution as shown below.

Electroelution of the purified JE virus protein: After the SDS-PAGE and staining, visualized viral protein on the gel was cut out and electrophoretically eluted into the running buffer diluted 1: 10 (Ziola and Scraba, 1976).

Western blotting: Proteins on the SDS-PAGE were electrophoretically transferred to nitrocellulose membrane according to Towbin *et al.* (1979) and Burnette (1981) with some modifications. The transfer was performed in the buffer containing 25 mM Tris, 192 mM glycine, 20 % methanol and 0.1 % SDS, pH 8.3, at 4°C for overnight at 8-10 V/cm in ETB-15 apparatus (Tohyoh Kagaku Sangyoh). The nitrocellulose membrane was briefly rinsed in deionized water and was inactivated by 3 % casein in PBS (phosphate buffered saline) containing 0.1 % NaN_3 for 45 min. The membrane was washed 3 times in PBS with 3 min intervals and was reacted with the first serum (hyperimmune anti-JE rabbit polyclonal serum) at 1: 1000 dilution in PBS containing 0.01 % NaN_3 at 37°C for 3 hours. The membrane was again washed as above and then reacted with peroxidase-conjugated anti-rabbit IgG sheep IgG at 1: 1000 dilution in PBS at 37°C for 2 hours. The membrane was washed as above and the antigenically active protein bands were visualized by incubation with the substrate solution of peroxidase (0.03 % 4-chloro-1-naphthol, 0.03 % H_2O_2 in PBS) at room temperature for appropriate time.

Chemicals: Acrylamide, bisacrylamide and 2-mercaptoethanol were the products of Wako Pure Chemicals Co. Hyperimmune anti-JE rabbit serum was kindly supplied by the Kanonji Institute, Research Foundation for Microbial Diseases, Osaka University. Peroxidase-conjugated anti-rabbit IgG (heavy and light chains) sheep IgG was obtained from Cappel Laboratories, USA. Nitrocellulose membrane type TM-2 was the product of Tohyoh Kagaku Sangyoh.

RESULTS

Fig. 1 shows SDS-PAGE of the electrophoretically purified JE virus envelope glycoprotein V3 (E) in lane 1, protein recovered from the combined phenol and interphase of JE virus after phenol extraction in lane 2, and molecular weight marker in lane 3. In lane 2 there are 2 major bands with estimated molecular weights of 70K and 54K daltons, respectively, with a minor component of 48K daltons. The 54K dalton component showed almost the same mobility as the purified V3 (E) protein obtained by the electroelution from the purified virus in lane 1, and was considered as possible V3 (E) protein recovered from the phenol and interphase. The reasoning was substantiated by the Western blotting experiment which was shown in Fig. 2. Here, anti-JE ploYclonal sera reacted almost exclusively with a single band of the specimen recovered from phenol and interphase after phenol extraction of the purified JE virus. The position of the band corresponded with the 54K dalton protein of the specimen.

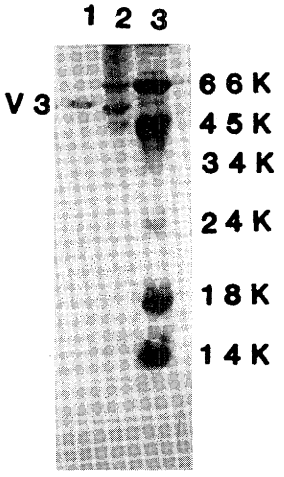

Fig. 1. SDS-PAGE of purified JE Virus V3(E) protein and proteins recovered from phenol -interphase after phenol extraction of the virus. Lane 1: V3 (E) protein electro-eluted from SDS-PAGE of JE virus. Lane 2: proteins recovered from phenol-interphase after phenol extraction of JE virus. Lane 3: molecular weight marker.

Fig. 2. Western blotting of the proteins recovered from phenol-interphase after phenol extraction of JE virus. The specimen was prepared and treated as described in the Materials and Methods and Text.

DISCUSSION

The result of our experiment showed that at least 2 major and one minor protein components were remaining in the combined phenol and interphase which were obtained after RNA was extracted from the purified JE virus preparation. The Western blotting experiment strongly suggested that 54K dalton component could be viral envelope glyco-

protein V3 (E) both by its molecular weight and reactivity with polyclonal anti-JE virus serum. Therefore, it appears that even after phenol extraction which could denature the protein, at least some of the antigenic epitopes of V3 (E) protein remained intact. The epitope(s) should have been immunogenically active when the JE virus was inoculated to rabbit in order to make antiserum and so could not have been newly exposed after denaturation by phenol extraction or SDS-PAGE. The 70K dalton component could possibly be residual bovine serum albumin which contaminated the virus preparation from fetal calf serum in the maintenance medium. We often found this component in JE virus preparations as shown in the previous report (Srivastava and Igarashi, 1985). The other minor component of 48K dalton, which was also shown in the JE virus preparation (Srivastava and Igarashi, 1985) could possibly be some cellular component.

As other members of flavivirus (Stollar, 1969; Trent and Quereshi, 1971; Westaway and Reedman, 1969), JE virus was shown to contain 3 structural proteins, V1, V2, and V3 (Shapiro *et al.*, 1971; Kitano *et al.*, 1974), however, present study showed definite reactivity of only possible V3 (E) protein with anti-JE serum. Coomassie Blue staining in Fig.1 suggests the presence of a faint band corresponding to the molecular weight of V2. However, the band did not react with polyclonal anti-JE serum, although fresh JE virus preparation showed the reactivity of V2 with the same polyclonal anti-JE serum in the Western blotting (data not shown). Therefore, it appears that whether antigenicity of V2 protein was inactivated after phenol extraction, or V2 (and possibly V1 also) was denatured such a way that it could not efficiently be solubilized from the protein precipitate into SDS and 2-mercaptoethanol before SDS-PAGE. The integrity of the recovered glycoprotein should be examined further for its fine structure of antigenicities and immunogenicities by monoclones and also at its molecular level as well.

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REFERENCES

1) Burnette, W. N. (1981): "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem., 112, 195–203.

2) Eagle, H. (1959): Amino acid metabolism in mammalian cell cultures. Science, 130, 432–437.

3) Gierer, A., & Schramm, G. (1956) Infectivity of ribonucleic acid from tobacco mosaic virus. Nature, 177, 702−703.
4) Hori, H., Morita, K., & Igarashi, A. (1986): Oligonucleotide fingerprint analysis of Japanese encephalitis virus strains isolated in Japan and Thailand. Acta Virol. in press.
5) Igarashi, A. (1978): Isolation of a Singh's *Aedes albopictus* cell clone sensitive to dengue and chikungunya viruses. J. Gen. Virol., 40, 531−544.
6) Igarashi, A., Kitano, H., & Fukai, K. (1963): An infectious and ribonuclease-sensitive fraction from mouse brain infected with Japanese B encephalitis virus. Biken J., 6, 21−23.
7) Kitano, T., Suzuki, K., & Yamaguchi, T. (1974): Morphological, chemical and biological characterization of Japanese encephalitis virion and its hemagglutinin. J. Virol., 4, 631−639.
8) Laemmli, U. K. (1670): Cleavage of structural protein during the assembly of the bacteriophage T4. Nature, 227, 680−685.
9) Nakamura, M. (1961): Infectious ribonucleic acids derived from mouse brains infected with 2 kinds of arbor virus group B. Nature, 191, 624.
10) Srivastava, A. K., & Igarashi, A. (1985): Comparison of Japanese encephalitis virus isolates from Japan and Thailand by peptide mapping. Trop. Med., 27, 269−275.
11) Stollar, V. (1968): Studies on the nature of dengue viruses. IV. The structural proteins of type 2 dengue virus. Virology, 39, 426−438.
12) Shapiro, D., Brandt, W. E., Cardiff, R. D., & Russell, P. K. (1971): The proteins of Japanese encephalitis virus. Virology, 44, 108−124.
13) Studier, F. W. (1973): Analysis of bacteriophage T7 early RNAs and proteins on slab gels. J. Mol. Biol., 79, 237−238.
14) Towbin, H., Staehelin, T., & Gordon, J. (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA., 76, 4350−4354.
15) Trent, D. W., & Quereshi, A. A. (1971): Structural and nonstructural proteins of Saint Louis encephalitis virus. J. Virol., 7, 379−388.
16) Westaway, E. G., & Reedman, B. M. (1969): Proteins of group B arbovirus Kunjin. J. Virol., 4, 688−693.
17) Ziola, B. R., & Scraba, D. G. (1976): Recovery of SDS-protein from polyacrylamide gels by electrophoresis into hydroxylapatite. Anal. Biochem., 72, 366−371.

フェノール抽出精製日本脳炎ウイルスからウイルスタンパクの回収
ASHOK KUMAR SRIVASTAVA, 五十嵐 章 (長崎大学熱帯医学研究所ウイルス学部門)
精製日本脳炎 (JE) ウイルスから RNA を抽出する目的でウイルスをフェノール抽出した際のフェノール層と中間層の混合物から JE ウイルス外被膜糖タンパクを回収した。このタンパクはドデシル硫酸ナトリウム存在下のポリアクリルアミドゲル電気泳動 (SDS-PAGE) で V3 糖タンパクと同一の移動度を示し JE に対する多価ウサギ抗血清を用いた Western blotting によって少なくともその抗原性の一部を保持している事が示された。この方法によって回収された糖タンパクの抗原性，免疫原性，及び構造について分子レベルでの検討を今後行う必要がある．