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Study on the Basic Morphology of Japanese Encephalitis Virus : The envelope and the origin of the large particles of hemagglutinin

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ABSTRACT : The fine structure in relation to the morphological and biological properties of associated antigens of Japanese encephalitis (JE) virion, particularly, the origin of large particles of hemagglutinin were studied in this paper. An envelope with projections was observed clearly in the electronmicroscopy of the preparation by double stain method using 1 percent phosphotungstic scid solution and 1 percent uranyl acetate solution. Large particles of hemagglutinin were observed when the purified virus was treated with α-chymotrypsin or when the virus was kept at 4°C for a long period after freezing and thawing several times. It was found that these particles having the hemagglutination property were constructed with the aggregation of several fine projections (hemagglutinins). The purified hemagglutinating and complement fixing components could be seperated from the disrupted virions by centrifnugation in the discontinuous gradient of cesium chloride. These components could be identified by electrophoresis in 10 percent acrylamide gels.

Many workers have reported that Japanese encephalitis (JE) virus revealed spherical shape with the envelope (40 to 50 nm in diameter) (Kitaoka and Nishimura, 1963, 1964; Fukai, 1970; Ota, 1965; Higashi, 1967; Oka, 1971; Hayashi, 1972; Kitano, Suzuki *et al.*, 1974). It is also a well-known fact that the mature JE virus carries the hemagglutinating property. Kitaoka and Nishimura (1963) and Fukai (1970) demonstrated the hemagglutinins associated with infectious virion and the other non-infectious hemagglutinins slow sedimented in sucrose density gradient centrifugation. In electronmicroscopic study, the non-infectious hemaggluinin revealed spherical particles measuring 16 to 18 nm in diameter (Fukai, 1970), however, there is still little knowledge about the production of these particles. In this paper, the basic morphological study on the envelope of JE virion, particulary, large hemagglutinating components are presented. Furthermore, the polypeptides of JE virus in relation to the biological properties of hemagglutinating and complement fixing components of the the virion are confirmed.

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MATERIALS AND METHODS

Virus

Nakayama NIH strain of Japanese encephalitis (JE) virus was used throughout the experiment. The passage history of that strain was not known in detail, but the strain underwent 41 passages in the suckling mouse brain in this laboratory.

Purification of the virus

Twenty percent emulsion of suckling mouse brain infected with JE virus in tris-saline pH 8.0 was centrifuged at 10,000 rpm for one hour (Refrigerator centrifuge, Tomy Seiko Co. Model RS-18p-2 Roter No. 3N). The supernatant was added with protamin sulfate drop by drop at a final concentration of 4 mg per ml while being shaken for 30 minutes in a ice bath. The specimen was centrifuged at 10,000 rpm for 30 minutes and the supernatant was treated with protamin sulfate repeatedly. The partially purified virus was added with carbon powder at a final concentration of 3 percent while being shaken for 30 minutes in a ice bath. The mixture was filtered with a millipore filter of 0.45 μ por-size. The filtrate was centrifuged at 100,000g for 4 hours (Spinco Model L2-65B Roter SW 50.1). The pellet was resuspended with tris-saline pH 8.0 in proportion of one tenth volume of original suspension and stored at -70°C before nse.

Hemagglutination and complement fixation test

The hemagglunation (HA) activity of purified virus and fractions obtained after cesium chloride density gradient centrifugation was performed with the microtiter technique modified from the method of Clarke and Cassals (1958). The complement fixation (CF) test was made by microtiter method modified from the method of Lennette *et al.* (1970).

Disruption of the purified virus

(1) The purified virus was mixed with Tween 20 at a final concentration of 1 percent and was shaken at room temperature for 30 minutes. The hemagglutinin and other components were released successfully from the virion by the treatment of Tween 20 without loss of their activities. These specimens were also used for the fractionation by CsCl density gradient centrifugation and the electrophoresis in acrylamide gels.

(2) The purified virus was kept at 4°C for one month or more after freezing and thawing at intervals of few weeks. It was aimed to release spontaneously the components from virions in the common refrigerator. These specimens were examined electronmicroscopically.

Treatment of the virus with α-chymotrypsin

(1) Purified virus suspension in tris-saline pH 8.0 and 1 mg per ml of α-chymotrypsin solution were mixed at the ratio of 1 : 1 in volume. The mixture was put on microgrids at certain intervals and dried in air. The specimens on microgrids were washed with drops of water and stained for observation with an electronmicroscope (JEM Model 100B).

(2) After the virus was treated with α-chymotrypsin, the specimen was centrifuged in discontinuous gradient of cesium chloride. The hemagglutinin in the fraction was identified by disc gel electrophoresis, because the specimen did not show hemagglutinating activity.

Cesium chloride (CsCl) density gradient centrifugation

The fractionation of purified or disrupted virus was performed by centrifugation in discontinuous gradient overlaid $\rho=1.450$, $\rho=1.350$ $\rho=1.210$ of CsCl solution containing 0.001 M EDTA at 100,000 g for 3 hours (Spince L2−65B, SW 50.1). The fractions were collected by 10 or 15 drops in each tubes.

Stain method for observation with electronmicroscope

The double stainning method was performed for the preparation of electronmicroscopic observation (Hayashi *et al.* 1972). Specimens on microgrids were dried in air and stained in turn with 1 % phosphotungstic acid (PTA) solution and 1 % uranyl acetate (UA) solution for 5 to 10 minutes respectively. Air drying and washing with water were performed in each interval of stanning process. Single stainning with 1 % UA was also performed for some specimens.

Acrylamide gel electrophoresis

Electrophoresis was done in 10 % (w/v) acrylamide gels cast to a height of 90 mm in 5 mm internal diameter perpex tubes. The gel and buffer systems were those described by Kennedy and Burke (1972). The gels in tubes were overlaid with the running buffer for a few hours before use. Specimens were added in proportion of one tenth volume of the mixture containing 2 % sodium dodecyl sulfate (SDS), 50 % glycerol and 4 % 2-mercapto-ethanol (2ME) solution. This mixture was boiled at 100°C for one minute and added with bromphenol blue (BPB) as the tracking dye. The running buffer was removed and the specimens were overlaid on the gels. Electrophoresis was performed at 8 mA per a tube for 8 to 10 hours and the gels were stained with coomassie blue (Maizel *et al.*, 1970).

RESULTS

1. JE virion enveloped with projection and empty virus

When the purified was stained doubly with 1 % PTA and 1 % UA solution described in methods, the spherical shape enveloped with projections was observed clearly as seen in photo. 2 and photo. 3, and the empty virus was observed as seen in photo. 4 and photo. 5. Average diameter of the virion was 45 nm ±2.5 nm, and the envelope of empty virus was measured as 14 to 18 nm in thickness.

2. Envelope

The sowellen envelope was observed in the specimen which was kept at 4°C for one month after freezing and thawing. Most of projections were released from the envelope. It was, however, recognized clearly that the projections attached on the basal membranous structure as seen in photo. 6. The intact envelope which was recognized as an empty virus was presented in photo. 4 and photo. 5. It was considered that the empty virus might vary easily in size as seen in photo. 4. The large particles having the hemagglutinating property could also be observed in this preparation (photo. 6).

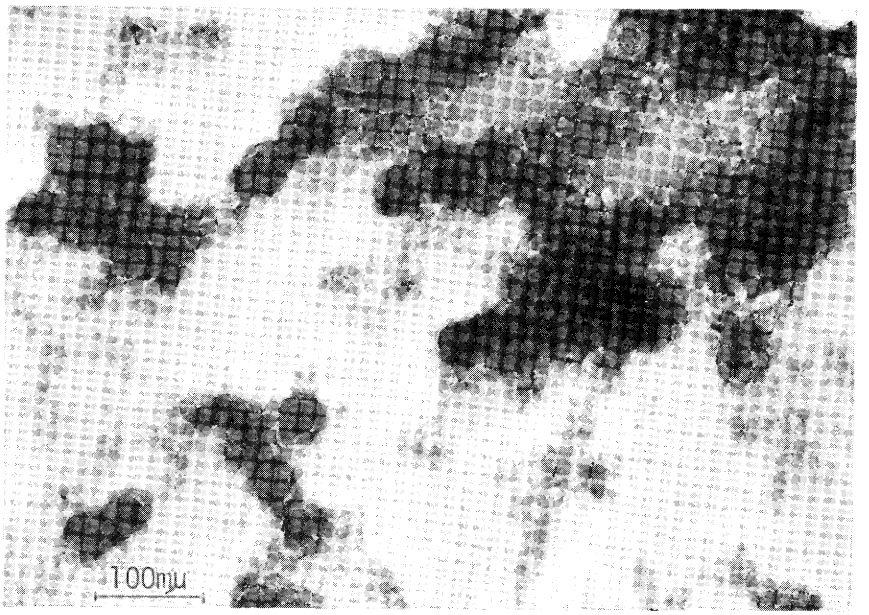


Photo. 1. Purified JE virus pariticles stained doubly with 1% PTA and 1 % UA solutions (magnification 1:140,000).

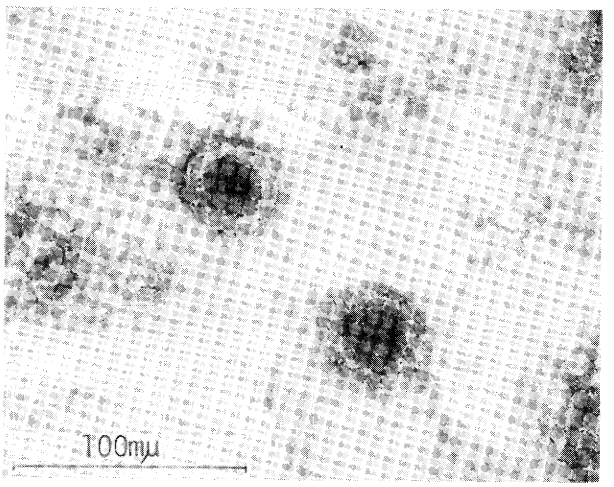


Photo. 2. JE virion stained doubly with 1% PTA and 1 % UA solutions (magnification 1:300,000).

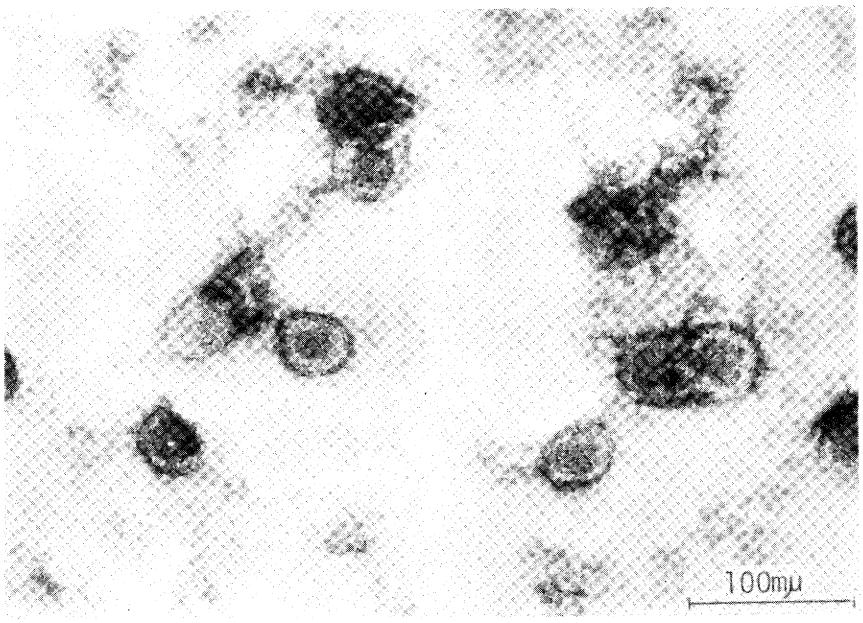


Photo. 3. JE virion stained doubly with 1% PTA and 1% UA solutions (magnification 1:210,000).

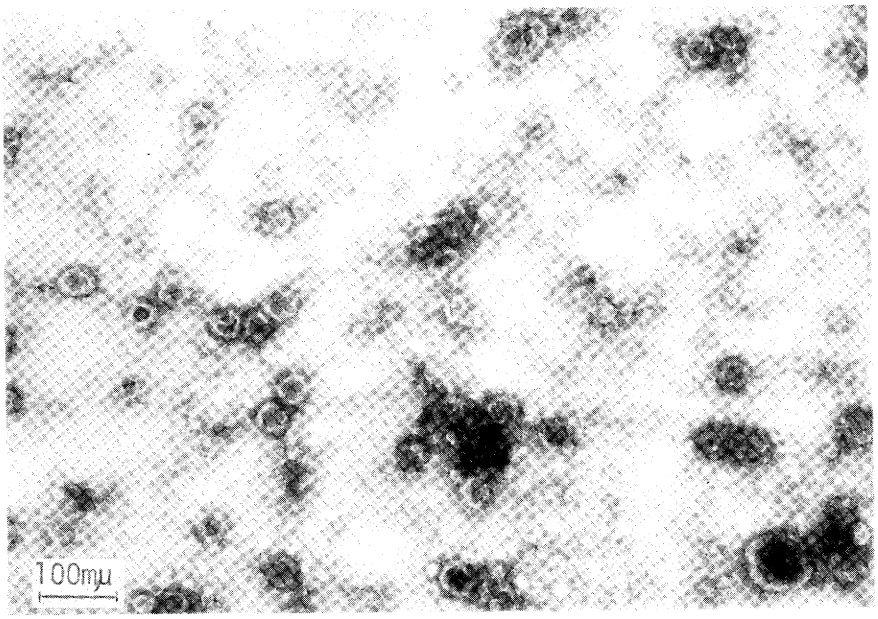


Photo. 4. Purified JE virus particles including empty virus stained doubly with 1% PTA and 1% UA solutions (magnification 1:100,000).

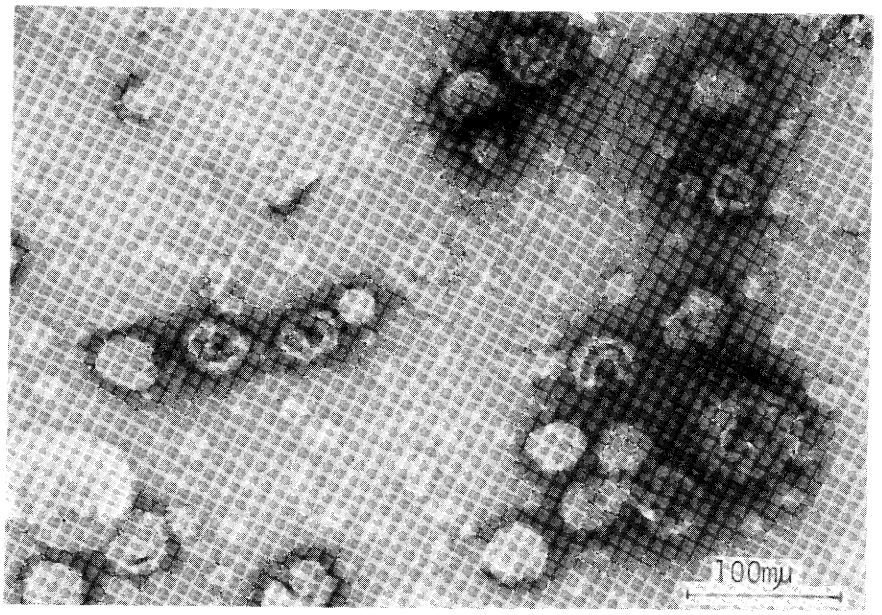


Photo. 5. Purified JE virus particles including empty virus stained doubly with 1% PTA and 1% UA solutions (magnification 1:200,000).

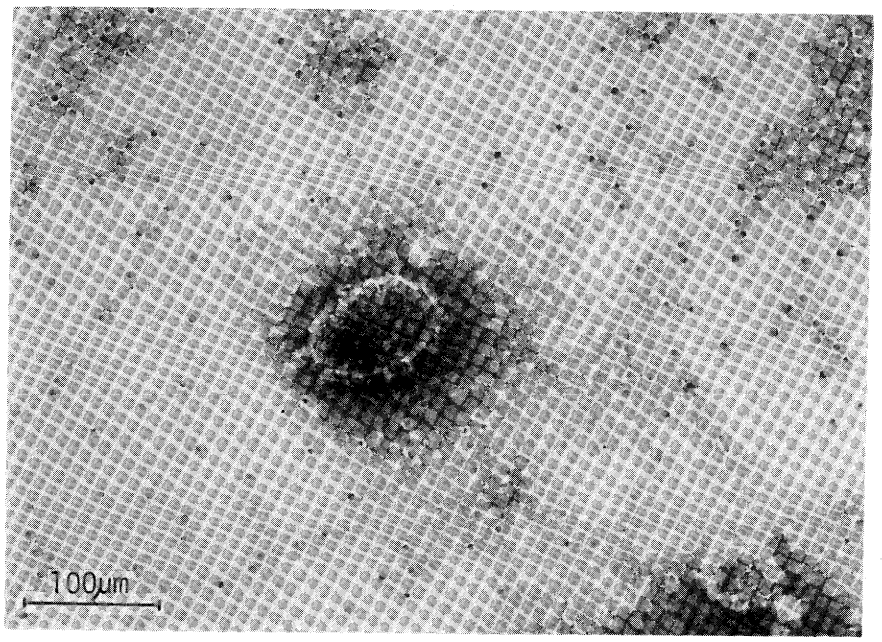


Photo. 6. Envelope is swollen after keeping the purified virus solution at 4°C for over one month after freezing and thawing. Specimen was stained doubly with 1% PTA and 1% UA solutions (magnification 1:175,000).

3. Projection (hemagglutinin) and large particles of hemagglutinating components (rapid sedimented hemagglutinin)

The purified virion easily released projections by freezing and thawing frequently as seen in photo. 7. The released projections were likely to aggregate together. The large particles having hemagglutinating property were often observed in the fresh preparation of purified virus suspension as seen in photo. 8. When the purified virus suspended in tris saline pH 8.0 was kept at 4°C for one month after freezing and thawing repeatedly, a large amount of the large particles having hemagglutinating property were observed as seen in photo. 9. These large particles were arranged along the rope-like aggregation of projections (photo. 10 and photo. 11). It was suggested that these large particles might be produced the aggregation of several or more projections. To make sure such a suggession, the purified virus was treated with α-chymotrypsin. As seen in photo. 12, photo. 13 and photo. 14, it was demonstrated that the large particles were constructed with the aggregation of several or more projections. These large particles were deprived of the hemagglutinating property by the treatment with α-chymotrypsin. However, they could be fractionated after centrifugation in the discontinuous gradient of CsCl similar to that indicated in Fig. 2 and Fig. 3. They were identified to be the hemagglutinins by the method of electrophoresis in 10 percent acrylamide gels as shown in the following data in this study.

4. Electrophoresis in acrylamide gels of virion, hemagglutinating and complement fixing components

When the disrupted virus treated with Tween 20 centrifuged in discontinuous density gradient of CsCl, the hemagglutinating and complement fixing components could be seperated as seen in Fig. 1. The hemagglutinating (Fr. 2 and Fr. 3 were combined) and complement fixing (Fr. 6 and Fr. 7 were combined) components were centrifuged again in discontinuous density gradient of CsCl. These components could be fractionated clearly into hemagglutinating (II-Fr. 1 and Fr. 2) and complement fixing (II-Fr. 5) components as seen in Fig. 2. The purified virus, hemagglutinating components (I-Fr. 2 and Fr. 3; II-Fr. 1 and Fr. 2) and complement fixing components (I-Fr . 6 and Fr. 7; II-Fr. 4 and Fr. 5) were examined for the analysis of structural polypeptides. The purified virus showed three major and three minor polypeptides. The three minor polypeptides could not be identified in connection with the function of the morphological situation of the virion (Fig. 3). The crude hemagglutinating components of the purified virions (I-Fr. 2 and Fr. 3) showed three polypeptides. However, when the hemagglutinating components were purified (II-Fr. 1 and Fr. 2), they showed a major polypeptide (Fig. 4 and Fig.5). Although the crude complement fixing components (I-Fr. 6 and Fr. 7) showed two polypeptides, the number was reduced to one after purification of the components (II-Fr. 4 and Fr. 5) as seen in Fig. 4 and Fig. 5. One of the polypeptides in the crude components corresponding to the middle major polypeptide of the purified virus (Fig. 3 and Fig. 4) was eliminated by the purification of these components (Fig. 5).

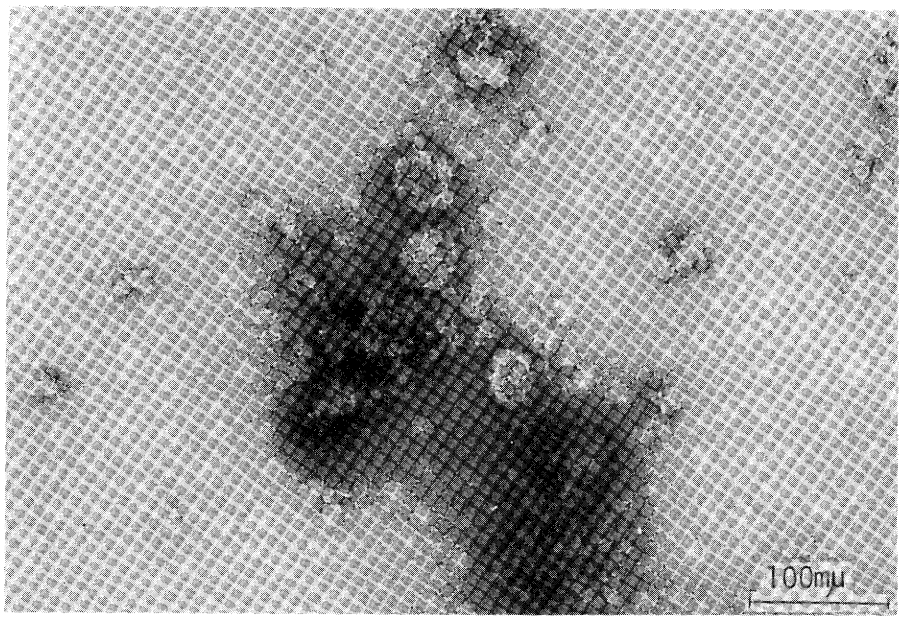


Photo. 7. JE virus particles releasing hemagglutinin components stained with 1% UA solution (magnification 1:175,000).

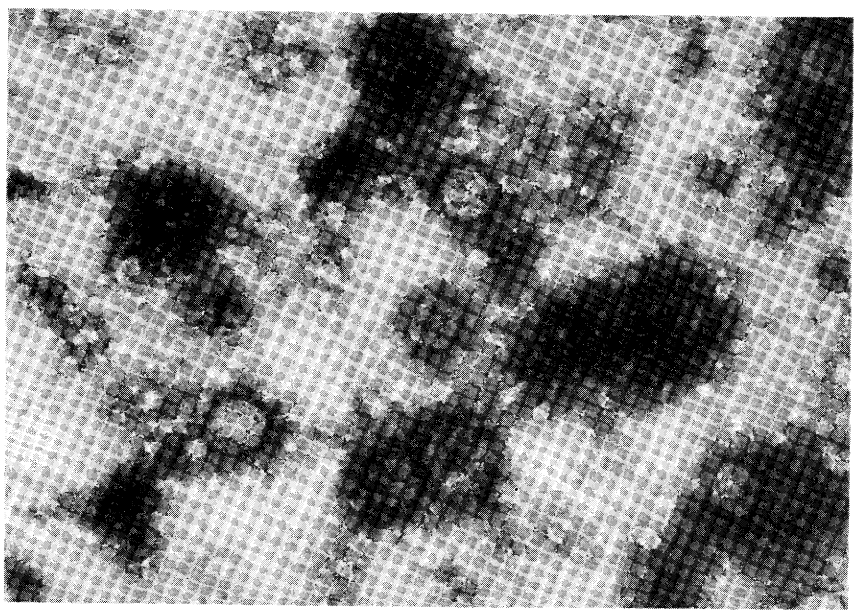


Photo. 8. Purified JE virus vaccine containing large hemagglutinin components stained doubly with 1 % PTA and 1 % UA solutions (magnification 1:175,000).

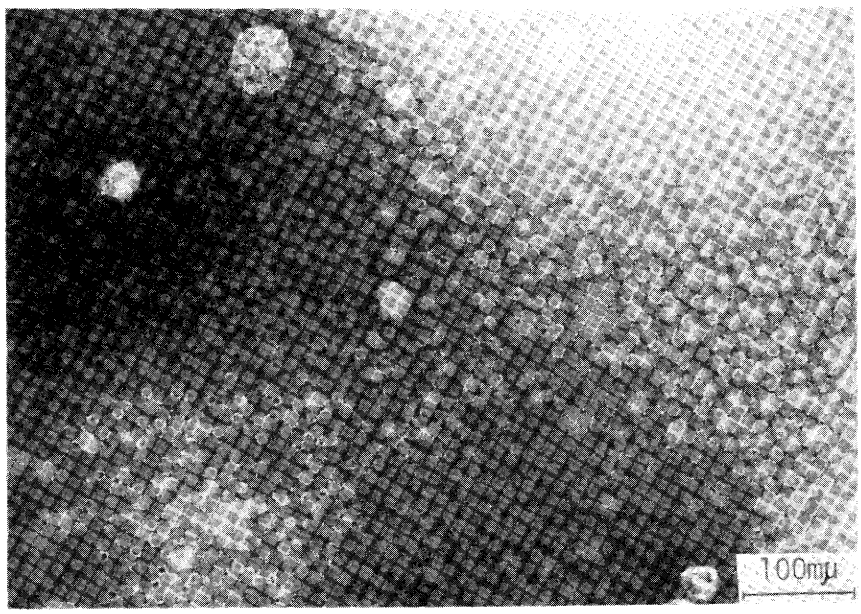


Photo. 9. Large hemagglutinin components observed in purified JE virus solution kept at 4°C for over three months after freezing and thawing frequently. Specimen was stained doubly with 1% PTA and 1% UA solutions (magnification 1:140,000).

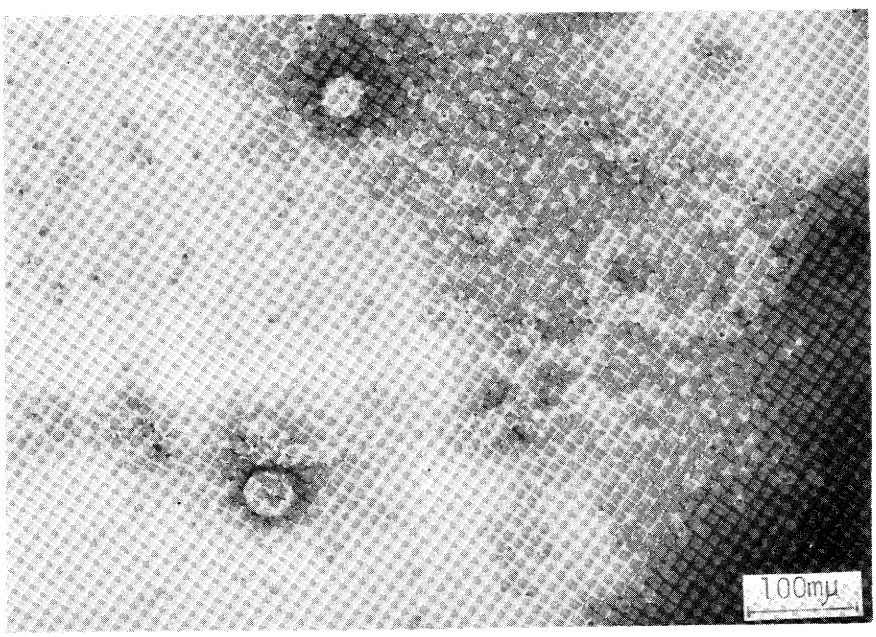


Photo. 10. Large hemagglutinin components observed in purified JE virus solution kept at 4°C more over three months after freezing and thawing frequently. Specimen was stained doubly with 1% PTA and 1% UA solutions (magnification 1:140,000).

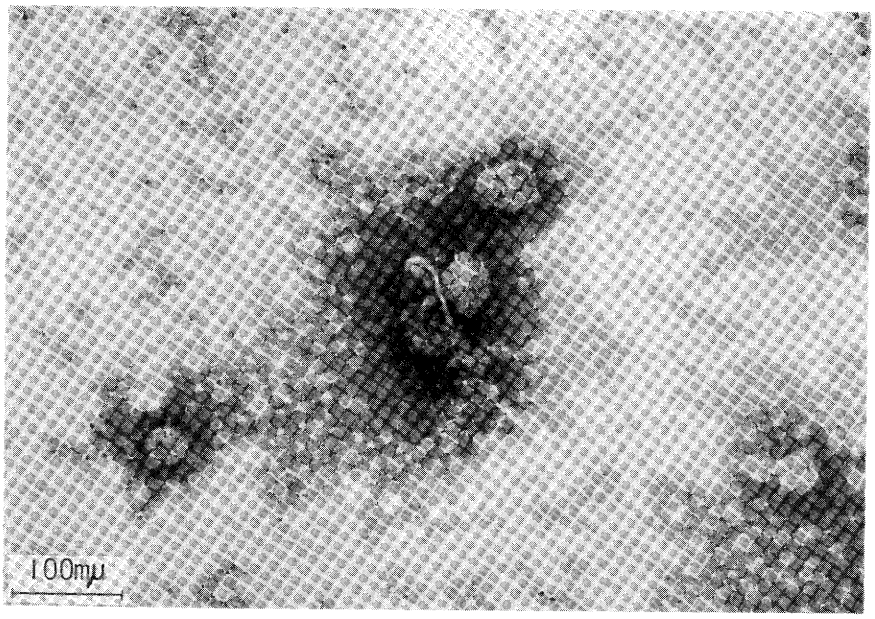


Photo. 11. Large hemagglutinin components and membranous structure observed in purified JE virus at the same time as seen in Photo. 9 and Photo. 10 (magnigication 1:140,000).

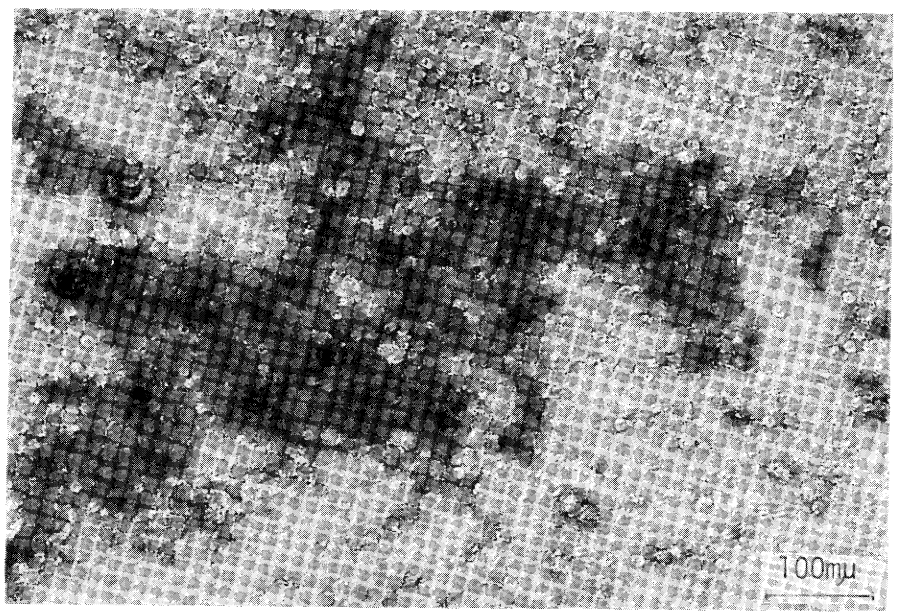


Photo. 12. Large hemagglutinin components produced by the treatment of purified JE virus with 1 % α-chymotrypsin and stained doubly with 1% PTA and 1% UA solutions (magnification 1:140,000).

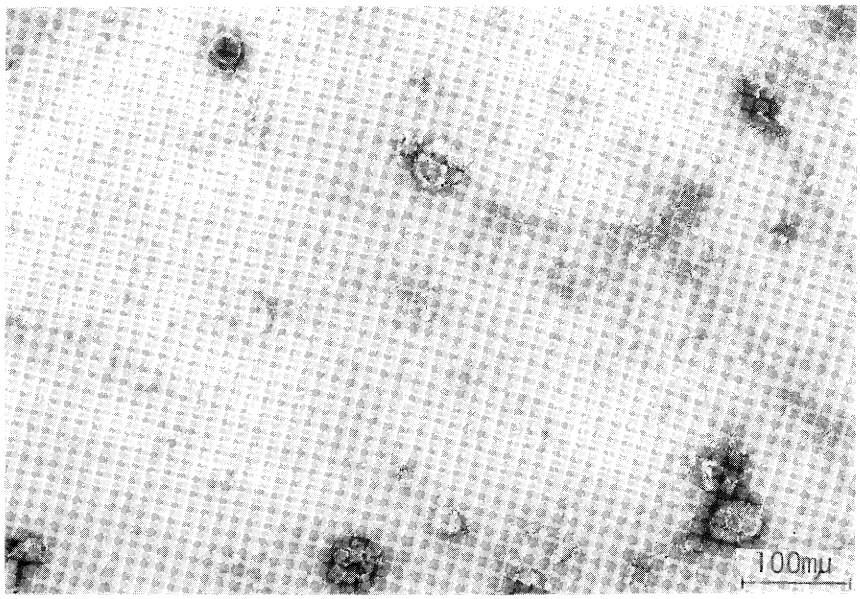


Photo. 13. Large hemagglutinin components produced by the treatment of purified JE virus with 1% α-chymotrypsin and stained doubly with 1% PTA and 1% UA solutions (magnification 1:140,000).

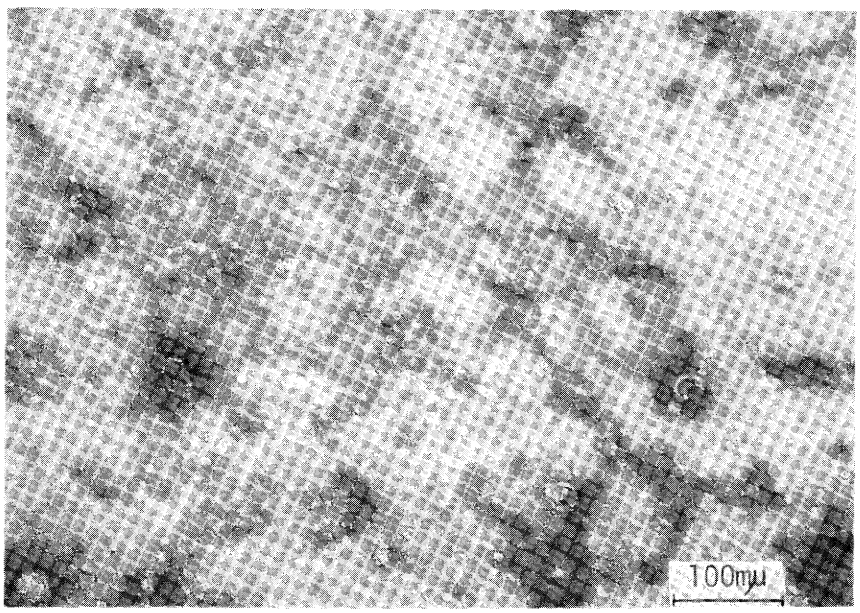


Photo. 14. Large hemagglutinin components produced by the treatment of purified JE virus with 1% α-chymotrypsin and stained doubly with 1% PTA and and 1% UA solutions (magnification 1:140,000).

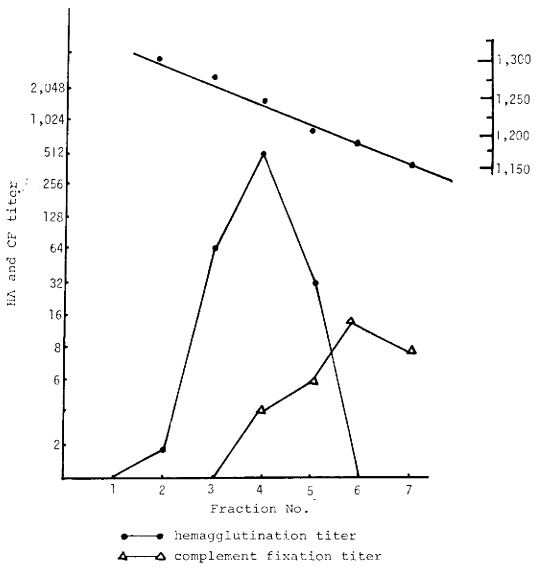
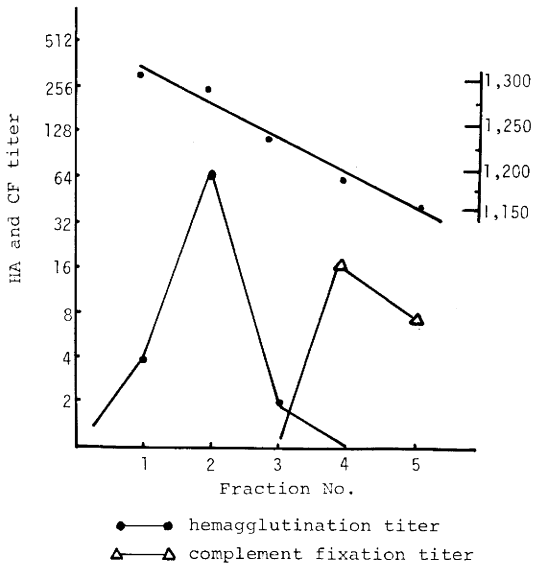


Fig. 1. Cesium chloride density gradient sedimentation of disrupted virion treated with Tween 20.



Fid. 2. Cesium chloride density gradient sedimentation of hemagglutinating and complement fixing Components.

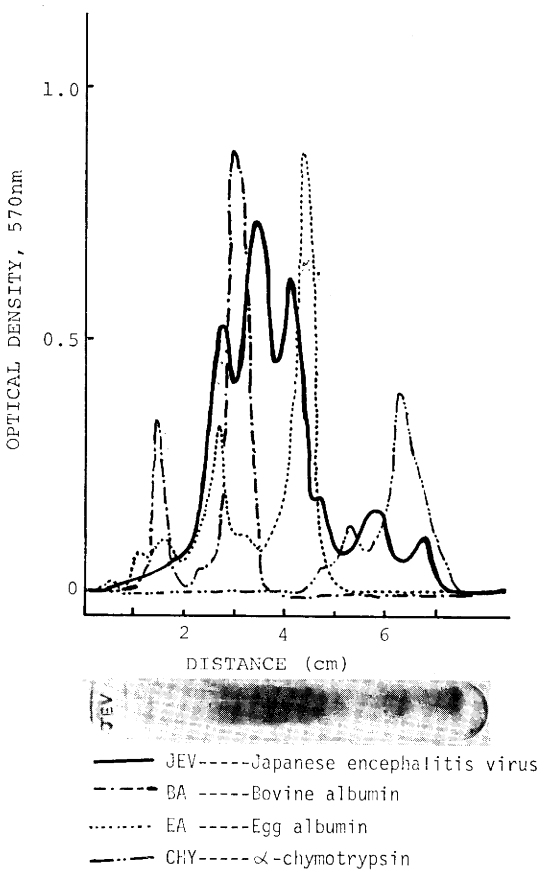


Fig. 3. Electrophoresis in 10 percent polyacrylamide gels of purified JE virus treated with Tween 20.

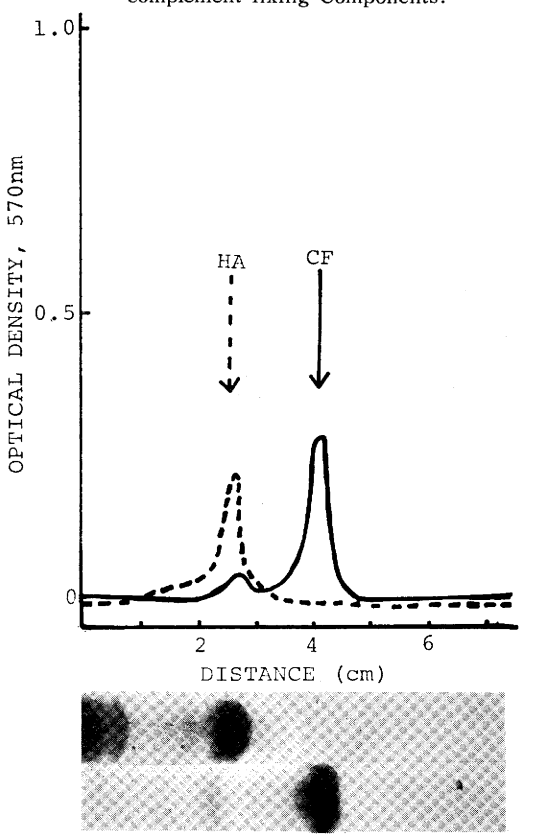


Fig. 4. Polypeptide of hemagglutinating (ρ= 1.290) and complement fixing components (ρ=1.180) in polyacrylamide gels.

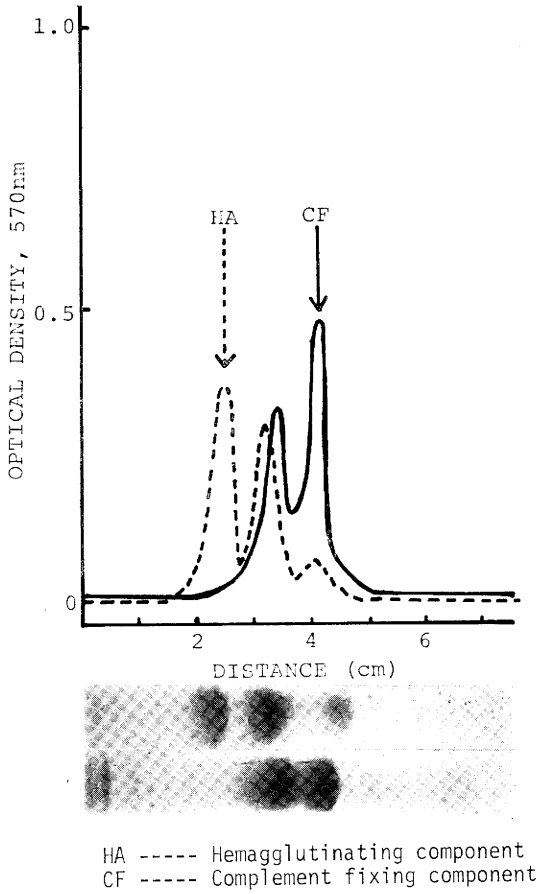


Fig. 5. Polypeptide of hemagglutianting (ρ= 1.310) and complement fixing components (ρ=1.160) in polyacrylamide gels.

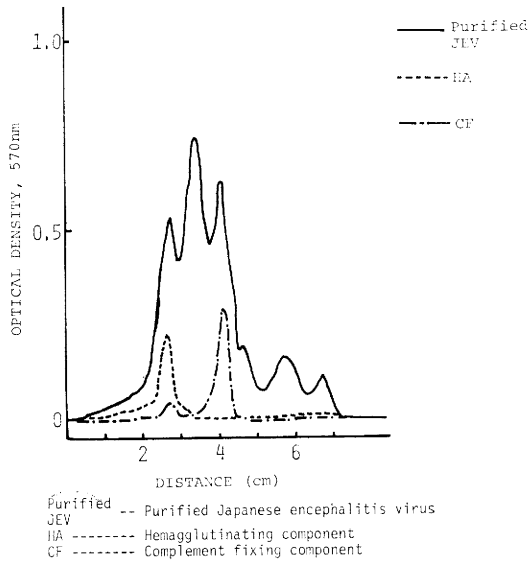


Fig. 6. Electrophoresis in acrylamide gels of hemagglutinating (ρ=1.310) and complement fixing (ρ=1.170) components of JEV.

DISCUSSION

Many workers reported that the virions of alpha-, flavi-viruses and bunyamwera group viruses presented a spherical shape surrounded by net-like structures. Horzinek (1973) states that "the projections may appear according to different techniques for the preparation of electronmicroscopic study ranging from a diffuse halo of filamentous to rod shaped structures likely as myxovirus spides", In fact, in this study, it was recognized that JE virions revealed the filamentous halo structure so far some particles appeared in photo. 1, 2 and 4 were concerned. In contrast, the virions were surrounded by net-like structure of fine projections as seen in photo. 3. Simpson *et al.* (1968) pointed out that arbovirus particles incompletely penetrated by PTA can easily be misconstrued as empty virus. However, strictly speaking, the empty shapes of JE virus missing their core occassionally appeared as seen in photo. 5. These empty shapes had not been illustrated in any preparations of togaviruses described by many previous workers. As shown in photo. 6, almost

projections were released from the envelope, however, the basal membranous structure attached with projections could be observed. This was most likely the inner layer of the envelope at the basis of projections, possibly the unit membrane described by Horzinek (1973) and Brown *et al.* (1973). On the other hand, it was considered that these membranous structures have a similarily to the findings that the pronase treatment of chikungunya virus resulted in smooth surface particles and these seemed to have a core surrounded by a membrane as reported by Igarashi *et al.* (1970).

Large hemagglutinating components measuring 16 to 18 nm in diameter were observed frequently in the fresh preperation of purified virus and also in the specimens kept at 4°C for a long period after freezing and thawing. These particles were also recognized by Nishimura *et al.* (1963), and a particular electronmicroscopic study was made on these components named Ht components by Fukai (1970). Ht components may be constructed with seven or nine subunits (Fukai, 1970), however, the origin of them in detail is still unknown. The treatment of purified JE virus particles with α-chymotrypsin resulted in the aggregation of projections forming large particles. It was demonstrated obviously that the large hemagglutinating particles were constructed with the combination of several or more projections (photo. 10, 12, 13, 14). Smith *et al.* (1970) described the doughnut forms of 7 nm diameter contained in the preparation of slow sedimented hemagglutinating components associated with dengue type 2 virions. Although their size was a half in diameter of the large hemagglutinating particles of JE virus as described above, it may be considered as a similar finding to the characteristics of the large hemagglutinating particles of JE virus. The hemagglutinating components (aggregated projections) and the large particles of them indicated the property of rapid sedimentation pattern by the centrifngation in CsCl density gradient as shown in Fig. 1 and Fig. 2. This evidence was different from the results obtained from the non-infectious hemagglutinin which sedimented slowly by sucrose density gradient centrifugation as reported by Kitaoka *et al.* (1963). It may be attributed to the different techniques for the centrifugation in discontinuous gradient of CsCl solution contained 0.001 M EDTA overlaid the disrupted virus solution containing 1.0−0.1% Tween 20. It was an obvious fact, however, that the hemagglutinating and complement fixing components could be clearly seperated in this study by CsCl density gradient centrifugation as seen in Fig. 2 and Fig. 5. The main three polypeptides of JE virus were studied by Shapiro *et al.* (1971), Igarashi *et al.* (1973) and Kitano *et al.* (1974). The study on the structural proteins should be explained in relation to the morphological situation of the components of the virions. In this study, it was attempted to confirm the polypeptides of purified hemagglutinating and complement fixing components. The JE virus preparation showed three major and three minor polypeptides as shown in Fig. 3. It was demonstrated that two of the three major polypeptides were derived from hemagglutinating and complement fixing components respectively. One of them was usually eliminated by the purification of the components (Fig. 4 and Fig. 5). This might possibly be a certain contaminated protein. The minor polypeptides could not be verified in this study. However, their significance may be elucidated in future studies.

SUMMARY

The basic morphological study on JE virus, particularly, the basal membrane of envelope and the origine of hemagglutinating large particles was carried out by means of electronmicroscopic observations. At the basic of projections, the membranous structure could be clearly observed. The hemagglutinating large particles were observed frequently in fresh or old preparation of JE virus by electornmicroscopy. It was demonstrated that these particles measuring 16−18 nm diameter were constructed with serveral or more projections. The hemagglutinating and complement fixing components showed a major polypeptide after purification respectively.

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日本脳炎ウイルスの形態学の研究: Envelope 及び赤血球凝集性大型粒子の生成由来について

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日本脳炎（日脳）ウイルス粒子の基本構造についてはなお残された問題が多い。特に，envelope の基本構造や赤血球凝集性大型粒子の由来については明らかでない。正常粒子は negative 染色法の改良によって小さい spike で蔽われている像がみられる。中心部の core を失い envelope のみ残された empty virus は明瞭な像として得られた。Spike が付着する envelope の基底は薄い膜様構造（basal membrane）であることを確認した。このような empty form や basal membrane は未だ記載がない所見である。日脳ウイルス精製材料に時に赤血球凝集性大型粒子をみることがあり，本粒子の生成由来については明らかでなかった。本研究で，粒子の自然崩壊と粒子を α-chymotrypsine で処理することにより，この大型粒子は spike が数個以上結合して出来ることを明かにした。従来，日脳ウイルス粒子から赤血球凝集性の構成成分と補体結合性の構成成分とをを明瞭に分画することが出来なかったが，本研究ではこれらを分画し，かつその構成蛋白を検査し，それぞれ一つの polypeptide から成っていることを明かにした。

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