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SUBJECT OF INVESTIGATION

The explanation of pathogenesis of Japanese encephalitis (JE) virus and the establishment of an attenuated strain of JE virus completely a virulent to man

Part III

Studies on Japanese encephalitis virus infection within cultivated animal cells by the application of fluorescent antibody technique

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THE EXPLANATION OF PATHOGENESIS OF JAPANESE ENCEPHALITIS (JE) VIRUS AND THE ESTABLISHMENT OF AN ATTENUATED STRAIN OF JE VIRUS COMPLETELY A VIRULENT TO MAN

PART III

STUDIES ON JAPANESE ENCEPHALITIS VIRUS INFECTION WITHIN CULTIVATED ANIMAL CELLS BY THE APPLICATION OF FLUORESCENT ANTIBODY TECHNIQUE

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C O N T E N T S

The explanation of pathogenesis of Japanese encephalitis (JE) virus and the establishment of an attenuated strain of JE virus completely a virulent to man

Part III

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a. Purpose of Study

Japanese encephalitis virus infection of mammals are knwon to result in a variety of sequences. A large part of infected animals does not show any sign of illness to severe involvement of the central nervous system. Originally, our study was attempted to know some fundamental rules of JE infection sequences occuring in the mammals.

Previous report, part II of the investigation under the same title, described the evidences which suggested the following infection process in the peripherally infected adult mice. Peripheral infection induces firstly the viral growth some where outside of brain tissue. Viremia ensues from the primary viral growth and provides a cause of viral invasion to brain tissue. Those two steps, primary viral growth and viremia seem to be essential form of JE infection of the adult mice. On the other hand, the reason why encephalitis occur in some mice and the other mice remain in apparetly healthy lookings is still quite obscure.

The next aim of our investigation was thought to clarify kinds of tissues or cells involved in the viral growth not only in the encephalitic phase but in the visceral stage. Recent development of fluorescent antibody technique seemed to provide a reasonable tool to approach to the present aim. However, no word has ever been done with this method for JE virus study.

Therfore, it was considered to be wise to begin with the simple system as possible, i.e. tissue culture system before applying it to more complicated tissues. Present paper deals with the results so far obtained in the cultivated cells infected with JE virus.

b. Materials and Methods

Virus strain: Nakayama-NIH strain of JE virus of repeated mouse brain passages more than hundred times was adapted to hamster kidney (HK) cells by approximately 10 passages was used.

Cultivated cells: Three kinds of cells, i.e. secondary transfered HK cells, continuous pig kidney (PS) cells and HeLa cells were employed. Those cells were reported to be highly susceptible to JE virus demonstrating cell degeneration (Kissling, 1957; Inoue et al, 1962; Buckeley, 1959). Cell suspensions of 1×10^5 cells per ml for HK and PS and 2×10^5 cells per ml for HeLa were seeded onto cover slips (12 x 32mm) kept in petri dishes. Dishes were incubated at 37°C for 2 days under the continuous supply of 5% CO_2 gas. Then, cells on cover slips were washed twice with phosphate buffered saline (PBS) and transfered to small square tubes with 0.9 ml of maintainance medium.

Growth media for cell culture: For HK and PS cells, 10% calf serum plus 0.5 La-Hanks BSS and for HeLa, 20% calf srum plus 0.1% yeast extract, 0.5% La-Earle BSS were used.

Maintainance medium: Four per cent calf serum free of JE antibody plus 0.5% La-Hanks BSS was used through all the experiment.

Inoculation and harvest: One tenth ml of infected HK fluid bearing JE virus titer of 10^7 $TCID_{50}$ per ml was inoculated into each 0.9 ml of maintainance medium of the experimental cell tubes. The input multiplicity was calculated to be between 5 to 10 so as to obtain

such condition that large number of cells were infected simultaneously. At suitable hours of infection described in the text, 2 tubes were randomly selected from the infected group and uninfected controls, and cover slips were taken out. They were washed with saline and Hanks BSS, then fixed in dried acetone at room temperature. Fixed preparations were dried on filter paper and kept at -20°C until antibody treatment.

Preparation of anti-JE virus antiserum: Five day chick embryo suspension infected with JE virus JaTH 160 strain was used for immunization of rabbits several times. Thus immunized rabbit serum was found to contain HI antibody 1:400 against 8 units of Nakayama HA antigen.

Preparation of conjugated anti-rabbit globurin Goat serum: Gamma globurin was purified from normal rabbit serum, mixed with Freund's adjuvant and injected into goat subcutaneously. Thus,obtained goat serum was conjugated with fluorescein isocyanate.

Staining and observation of the specimen: Anti-JE virus antiserum was diluted to 1:20 and mounted on the fixed cells on cover slips for 30 min at room temperature. Cover slips were then washed with saline and mounted with conjugated serum for another 30 min. Afterwashing off serum, cells on cover slips were observed by Reichert's fluorescent microscope.

C. Experimental Results

1. Infection of HK cells.

Cells of uninfected controls were stained by fluorescein faint and diffuse both in cytoplasm and nucleus (Pl. 1 and 2). It was obvious at our present experiment that some non-viral or non-specific staining

was unavoidable. However, it should be emphasized that there was no difference in the illumination of cytoplasm and nucleus. At 2, 6 hours of infection, no difference could be observed with cells between infected and uninfected groups. Remarkable difference was at first noted at 10 hrs of infection. As clearly shown in Plates 3 and 4 marked illuminated boundary was observed around the nucleus in some cells. In contrast to the above boundary, the nucleus appeared as a dark spot. At 15 hrs., bright area expanded in cytoplasm from perinuclear site. Illuminated area seemed diffuse and consisted of many fine granules. Number of illuminated cells became more increased. Whole part of cytoplasm became stained in most of cells after 24 hours of infection (Pl. 5). Granules in the illuminated area appeared more coarse than before. It should be pointed out that the nuclei were still remained unstained. At this period, cells began to indicate degenerative changes, i.e. projection, shrinkage and leakage of cytoplasm. Coincidently with cytopathic changes, viral infective titer in the culture fluid increased abruptly.

2. Infection of PS cells.

In the earlier stage of infection, infected cells were stained by fluorescent faint and diffuse (Pl. 7) and no difference could be observed between infected and control cells (Pl. 6). It was also pointed out that little difference was noted in the brightness of nucleus and cytoplasm of the cell. Appearance of the infected cells differed remarkally at first 10 hours after the virus inoculation (Pl. 8). Cytoplasms of some part of infected cells began to emit remarkable fluorescent light. Loci of the illuminated areas were restricted to the perinuclear area. However, it did not appear as a sharp boundary observed in HK cells.

Number of the illuminated cells increased at 15 hours (Pl. 9). At this time, most of cells seemed to contain bright area. Within a single cell, bright area consisted of fine granules occupied the whole cytoplasm. The fact should be emphasized that nucleus remain dark in quite a contrast to the cytoplasm. No degenerative process could be observed by this time. Cytophathy was noticed from 24 hours of infection. At this stage, coarse particles were marked in the light scattering area in cytoplasm (Pl. 10). Though some illuminated material could be seen in nucleus, it would be explained by cytoplasmic materials covering the top of the nucleus.

3. Infection of HeLa cells.

Pictures obtained with HeLa cells were essentially the same with those observed with PS cells. Only the difference we noticed between above two cell systems was the fact that the infection proceeded slower in HeLa than in PS. First recognition of specific viral antigen in HeLa cell was at 15 hours after the inoculation as seen in Plate 12. Control cells shown in Plate 11 will give the idea that non specific staining seemes a little stronger in HeLa. However it should be kept in mind that exposing time with control specimen was 2 to 3 times longer than that with other infected pictures. Most of cells became illuminated at 24 hours and lasted without sign of degeneration for more than 24 hours (Pl. 13,14). Antigen accumulation was restricted to cytoplasmic area and nucleus seemed completely unstainable. Degeneration of cells appeared 72 hours of infection. Illuminated area seemed to localize within the cytoplasma and coarse particles became remarkable at this stage (Pl. 15).

d. Discussion

The purpose of the present investigation is to know the applicability of fluorescent antibody technique to JE virus-cell system and to know the general rule of viral antigen accumulation inside the cell. It has been known with tissue culture of JE virus that there are two types of cells susceptible to JE virus. The one shows degenerative changes by the infection and another appears completely unaffected, though both types of cells render JE virus to grow inside the cells. HK and PS cells were known to belong to the former cytopathogenic type. However, the opinion has not been in accord with HeLa cells. Scherer and Syverton (1954) and McCollum and Foley (1957) reported inconsistent cytophathic effect with HeLa cells infected with JE virus. On the contrary, Buckeley (1959) reported consistent cytopathic effect with this systim. The latter author seemed to attribute this difference to the difference of the medium in which cells were propagated. However, HeLa cells which were used in the present experiment was found highly degenerative after the JE virus infection. Thus, cytopathic changes enabled us viral titration on this cell line. Therefore, three kinds of cells used in the present experiment showed eventually cytopathic changes.

On the site of antigen production of JE virus inside the cell. Prince (1960) suggested some participation of cell nucleus observing specific fluorescent illumination inside the nucleus at earlier stage of infection to HK cells. On the contrary, Noyes (1955) described no evidence of antigen localized in the nucleus of mouse cortex or human carcinoma cells infected with West Nile virus which was antigenically retated to JE virus. In the present report, JE antigen was found strictly in cytoplasm at every stage of

infection and no evidence was obtained whatsoever to suggest the localization of JE antigen within cell nucleus similarly to Noyes's report. Nevertheless, it would not be correct to conclude that cell nucleus has no relation to antigen production of JE virus. Provided that our observation of the illuminated perinuclear boundary is specific for viral antigen, it seems still probable that cell nucleus may take some part for the initiation of viral antigen reproduction. Although such marked boundary could not be found in cases of PS and HeLa infections, it should be emphasized that no antigen producing site could be observed completely independent to the nucleus in the earlier stage of infection.

As a whole, the pictures elucidated in the present report seems quite similar to those reported in West Nile, Dengue(Buckeley, 1961) viruses suggesting the existence of common rule in the reproduction of arbor viruses. It was also confirmed that fluorescent antibody technique could furnish a convenient tool for the investigation of pathogenesis of JE virus.

e. References.

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Prince, A.M. 1960. personal communication.

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f. Abstract of the Final Report.

Process of Japanese encephalitis virus infection in cultivated animal cells, i.e. hamster kidney, porcine kidney and HeLa cells was followed by fluorescent antibody technique. The infection was adjusted to obtain multiplicities 5 - 10 for each system. Viral antigen was firstly detected in perinuclear cytoplasm at 10 - 15 hrs of infection, then numbers of the antigen producing cells increased and antigen producing area expanded within cytoplasm. No evidence was obtained that antigen localized in nucleus. Infected cells were eventually degenerated in all cell systems. Applicability of fluorescent antibody technique to the investigation of pathogenesis was considered hopeful.

Explanation of Plates

Plate 1. Uninfected control of HK cells. 200 X. Exposure: 20".

Plate 2. Uninfected control of HK cells. 500 X. Exposure: 30".

Plate 3. HK cells at 10 hrs after the inoculation. 200 X. Exposure: 30".

Plate 4. HK cells at 10 hrs after the inoculation. 500 X. Exposure: 30".

Plate 5. HK cells at 24 hrs after the inoculation. 200 X. Exposure: 10".

Plate 6. Uninfected PS cells. 200 X. Exposure: 80".

Plate 7. PS cells at 6 hrs after the inoculation. 200 X. Exposure: 40".

Plate 8. PS cells at 10 hrs after the inoculation. 200 X. Exposure: 45".

Plate 9. PS cells at 15 hrs after the inoculation. 200 X. Exposure: 45".

Plate 10. PS cells at 27 hrs after the inoculation. 200 X. Exposure: 30".

Plate 11. Uninfected HeIa cells. 200 X. Exposure: 120"

Plate 12. HeLa cells at 15 hrs after the inoculation. 200 X. Exposure: 60".

Plate 13. HeLa cells at 24 hrs after the inoculation. 200 X. Exposure: 60".

Plate 14. HeLa cells at 48 hrs after the inoculation. 200 X. Exposure: 30".

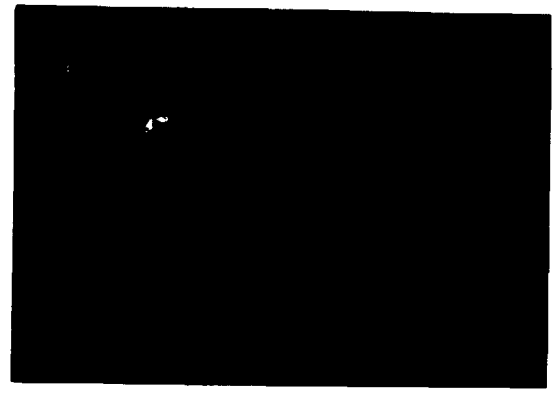


Plate 1

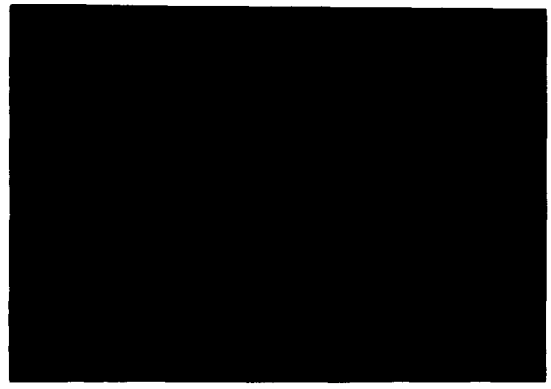
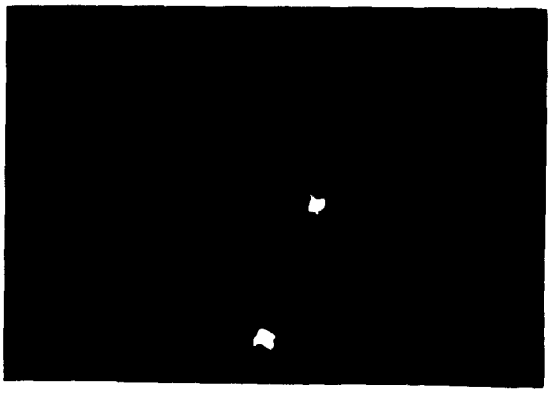


Plate 2

Plate 3

Plate 4



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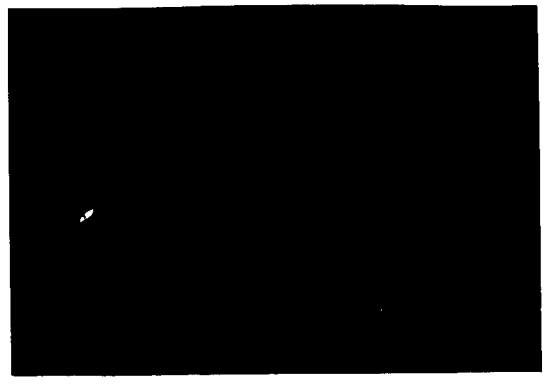

Plate 6

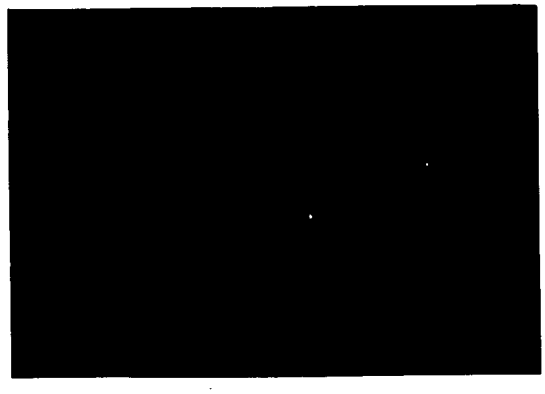

Plate 7

Plate 8

Plate 9

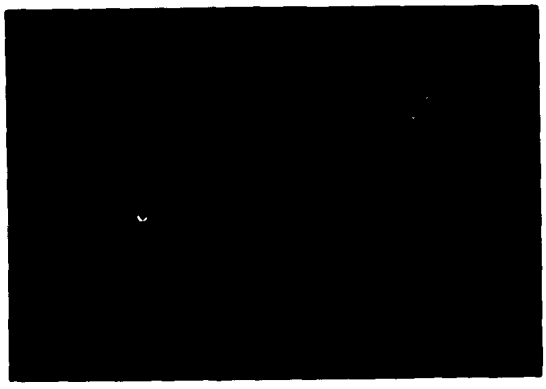

Plate 10

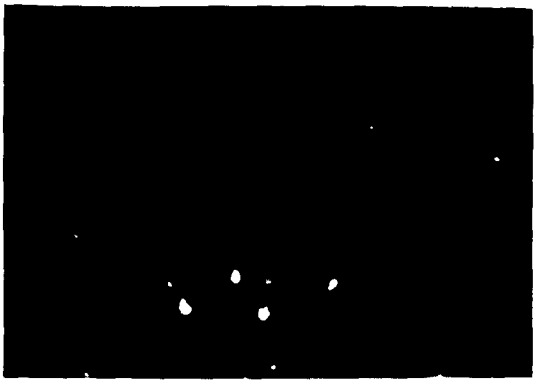

Plate 11

Plate 12

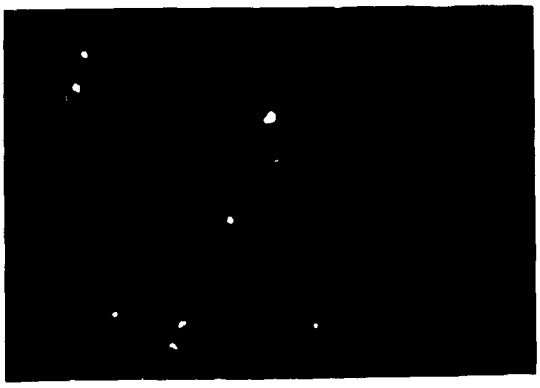

Plate 13

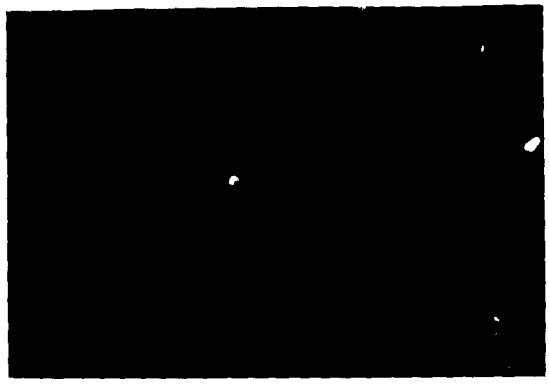

Plate 14

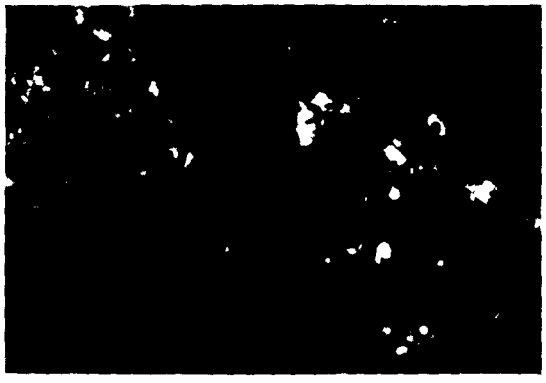

Plate 15

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National Institute of Health, Tokyo (Japan)
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Final Report No. 1, 1 Feb 62 to 31 Jan 63. 9 p. incl illus, tables, 8 refs.
(Contract DA 92-557-FEC-35805) Unclassified report

Process of Japanese B encephalitis virus infection in cultivated animal cells, i.e. hamster kidney, porcine kidney and HeLa cells was followed by fluorescent antibody technique. The infection was adjusted to obtain multiplicities 5-10 for each system. Viral antigen was initially detected in perinuclear cytoplasm at 10 - 15 hrs of infection, then numbers of the antigen producing cells increased and antigen producing area expanded within cytoplasm.

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1. Encephatitis Viruses

I. Title: None
II. Oya, Akira
III. U.S. Army Research and Development Gp (FE), OCRD, DA, Wash, D. C.
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Encephalitis Viruses
Antigens
Mice
Japan
Viability
Separation
Antibodies
Infections
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