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Virological Studies on Measles Virus

I. Isolation of Measles Virus Using FL Cells and Immunological Properties of the Isolated Agents

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SUMMARY

Two agents were isolated from throat washings of measles patients in FL cells. One of them, named Toyoshima strain, was identified as the agent of measles by immunological and morphological studies.

The difference between Edmonston strain and this strain could not be detected by immunological and cytopathogenic properties.

INTRODUCTION

Several reports have been published on the isolation of measles virus using primary cultures of various tissues (Cohen *et al.*, 1955; Ruckle, 1957; Ruckle and Rogers, 1957; Bech and von Magnus, 1958; Mutai, 1959) since the first successful isolation by Enders and Peebles (1954). Attempts to isolate this virus in HeLa and Hep-2 cultures were unsuccessful (Bech and von Magnus, 1958).

Many attempts had been made in our laboratory to isolate measles virus in monkeys and in embryonated eggs (Taniguchi *et al.*, 1954 a, 1954 b and 1956), but it was rather difficult to identify and maintain the isolated agents.

The present study is concerned with the isolation of measles virus using established tissue culture cells of FL strain and the immunological properties of the isolated agent.

MATERIALS AND METHODS

1. *Specimens for isolation*

Sera or throat washings of measles patients were collected shortly before or after the onset of rash. Washings are centrifuged twice at about 2,000 rpm for 20 minutes. Within a few hours after their collection, 0.2 or 0.3 ml of the materials were inoculated into two tubes containing FL cells.

2. *Tissue culture cells*

Established human amnion cells of FL strain were used for the isolation and the titration.

Monkey kidney cells were prepared by Younger's method (1954) for fluorescent antibody staining.

Slide cell cultures were prepared for morphological observations.

3. *Medium*

For growth medium of FL cells Earle's balanced salt solution containing 0.5 per cent lactalbumine hydrolysate (LE) with 15 per cent ox serum was used. For maintenance medium LE with 3 per cent ox serum was used.

4. *Serum*

Sera from measles patients at various stages and from children who had no history of measles were collected for serological tests. Anti-Edmonston human serum was prepared in this laboratory (Okuno et al., unpublished data) and anti-Edmonston monkey serum was kindly given by Dr. Matsumoto and Dr. Mutai (The Institute for Infectious Diseases, Tokyo University). All sera were heated at 56°C for 30 minutes and stored at -20°C.

5. *Measles virus*

Edmonston strain kindly given by Dr. Matsumoto and Dr. Mutai was used.

Titration: TCID₅₀ was determined as follows. Ten fold serial dilutions of the material were prepared. Three tubes of FL cells were inoculated respectively with 0.2 ml of each diluent and after one hour's incubation at 37°C, one ml of maintenance medium was added to each tube. Maintenance medium was changed several times during the observation period. After two weeks' observation, the TCID₅₀ was calculated by Reed and Muench's method.

6. *Neutralization test*

Serial two fold dilutions of serum were prepared. Diluents were added with equal volumes of the isolated agent containing about 100 TCID₅₀/ml. Mixtures were placed in an ice bath for one hour, then three tissues per each dilution were inoculated with 0.2 ml of the mixture. The antibody titer is expressed as the highest serum dilution which showed complete neutralization of the agent after two weeks' observation.

7. *Complement fixation test*

The test was performed according to Kolmer's method.

Infected tissue cultures were frozen when maximum cytological changes appeared and stored at -20°C for the CF-test. As the hemolytic system, sensitized ox red cells were employed.

8. *Fixation and staining*

Slides fixed with Bouin's fixative were stained with hematoxylin and eosin. Those fixed with methanol were stained with Giemsa's solution.

9. *Fluorescent antibody technique*

The indirect method described by Weller and Coons (1954) was employed using anti-Edmonston human serum and anti-human-globulin rabbit immune serum conjugated with fluorescein-isothiocyanate.

Specificity of the staining was established as follows: i). Uninfected cultured cells revealed no fluorescence by the usual treatment. ii). When anti-Edmonston serum was replaced by acute phase serum of measles, no fluorescence was visible in the infected cells. iii). In case when the conjugated antibody was applied directly for the infected cells, no distinct fluorescence could be seen.

RESULTS

1. *Isolation of the agents*

7 sera and 12 washings were collected from 15 patients. One serum and 7 washings showed cytopathogenic effects 7 to 16 days after the first inoculations. When cytopathogenic effects became evident, cell-fluid suspensions were made and 0.2 ml of each sample was inoculated in duplicate into new tissue cultures. Tubes

which failed to show any cytological change were passed to new cultures at the 16th day of the first passages. Few samples showed cytopathogenic effects in consecutive passages. After several passages no obvious cytological changes could be seen within the observation period. 10 paired tubes, which had represented no prominent cytological change, were treated with EDTA, and the cells were removed from the tubes at the end of the observation period. After a brief centrifugation at 500 rpm, cells of each pair were resuspended in 3 ml of maintenance medium and cultivated in three tubes at 37°C.

Table 1. Passages of the agents which showed cytopathogenicity in the first inoculations.

Passages Specimens No. Material		1	2	3	4	5	6	7
1	W	+	+	+	+	—T.	—T.	—
2 *	W	+	+	+	?	?T.	+	+
4	W	+	?	+	—T.	—T.	—	
5	W	+	+	—T.	—T.	—		
6	B	+	—T.	—T.	—			
7	W	+	?	+	?T.	+	+	
8	W	+	+	—T.	—T.	—		
14	W	?	?	—	—T.	—T.	—	

+: Cytopathic changes present.

—: Without cytopathic change.

W: Throat washings.

B: Serum.

T: Treated with EDTA and passed to next passage.

*: Toyoshima strain.

5 and 6 specimens were collected simultaneously from one patient.

After 2 and 3 days of cultivation, conspicuous cytological changes were observed in all tubes of two samples. 8 other samples showed no cytological changes within the two weeks' observation period and in consecutive passage with the treatment described above. One of the two positive samples, named Toyoshima strain, showed striking cytopathogenic effects within a few days after the subsequent inoculation into new FL cultures. TCID₅₀ of this agent at the 7th passage in FL cells was 10^{6.5}/0.2 ml.

2. Immunological properties of Toyoshima strain

a. Neutralization test:

Four human sera were used for neutralization tests. They were collected from an acute phase measles, a convalescent phase measles, a child having no history of this diseases and a convalescent phase of a case of measles of experimental infection (Edmonston strain).

As shown in Table 2 Toyoshima strain was neutralized by convalescent serum and anti-Edmonston serum and not by acute phase or uninfected sera.

Table 2. Neutralization of Toyoshima strain with human sera.

Serum	Convalescent	Anti-Edmonston	Uninfected and Acute phase
Titer	$\times 64$	$\times 64$	$< \times 4$

48 TCID₅₀/a dilution of the agent was inoculated.

b. Complement fixation test

Complement fixing antigen of Toyoshima strain and of Edmonston strain were tested with one convalescent and one anti-Edmonston monkey sera. As shown in Table 3 a and b, considerable similarity was observed between the strains.

Table 3 a. Comparison of CF-antigenicity of Edmonston and Toyoshima strains with convalescent human serum*.

		Edmonston strain						
Serum dilution		20	40	80	160	320	640	1280
Antigen dilution		—	—	—	(Serum control)			
1	±	+						
2	—	+	+	+	+	+	±	—
4	—	+	+	+	±	—	—	—
8	(Antigen control)	+	±	±	—	—	—	
16		±	±	—	—	—	—	
32		—	—	—	—			

		Toyoshima strain						
Serum dilution		20	40	80	160	320	640	1280
Antigen dilution		—	—	—	(Serum control)			
1	±	+						
2	—	+	+	+	+	+	±	—
4	—	+	+	+	±	—	—	—
8	(Antigen control)	+	+	+	—	—	—	
16		±	±	±	—	—	—	
32		—	—	—	—			

* : Serum 17 days after the onset of rash.

Table 3 b. Comparison of CF-antigenicity of Edmonston and Toyoshima strains with anti-Edmonston monkey serum.

		Edmonston strain						
Serum dilution		10	20	40	80	160	320	640
Antigen dilution		(Serum control)						
1	—	+						
2	—	+	+	+	+	+	—	—
4	—	+	+	+	±	—	—	—
8	(Antigen control)	—	—	—	—	—	—	
16		—	—	—	—	—		
32		—	—	—	—			

		Toyoshima strain						
Serum dilution		10	20	40	80	160	320	640
Antigen dilution		(Serum control)						
1	—	+						
2	—	+	+	+	+	+	±	—
4	—	+	+	+	+	±	—	—
8	(Antigen control)	—	—	—	—	—	—	
16		—	—	—	—	—		
32		—	—	—	—			

The correlation between the development of CF-antibodies to Toyoshima strain and the days after the onset of disease was studied (Fig. 1). As the antigens of the tests, pooled FL cells infected with Toyoshima strain were used at three fold dilution after freezing and thawing. CF-antibodies were first detected a few days after the onset of the rash, and 16 to 20 days after the onset of the disease the titer of antibodies reached their maximum. No CF-antibody could be detected in three samples collected before and at the onset of the rash. Sera from nine children who had no history of the disease were tested. They had no detectable CF-antibody, with one exception.

c. *Morphology:*

FL cells infected with Toyoshima strain showed remarkable cytopathological changes with giant cell formation. (Fig. 2). Cytoplasmic inclusion bodies were readily detectable in hematoxylin-eosin stained preparations (Fig. 3). Intranuclear inclusion bodies were also detected (Fig. 3). Both inclusions were eosinophilic and Feulgen negative and their characteristics were quite similar to the description by Black *et al.* (1959).

Several reports have been published on the isolation of measles virus using various primary cultures. Attempts to use HeLa cells and Hep-2 for isolation have been unsuccessful (Bech and von Magnus, 1958). Neither in our laboratory succeeded its isolation using HeLa cells. Recently we were informed in a personal communication of the successful isolation of measles virus using FL cells by Dr. Mutai. In our experiment, two agents could be isolated using FL cells from throat washings of acute phase measles patients. No agent could be established from serum samples. In some cases of unsuccessful isolations, cytopathological changes and a few cytoplasmic inclusion-like bodies could be observed in the 1st or the 2nd passage, suggesting that FL cells might be infected with the agent from the patient, but its adaptation to this cell might be rather difficult. The number of samples which showed cytopathic changes decreased remarkably in early passages. Even in the successful cases, the changes in the cells were once indistinctive and the presence of the agent could not be expected. Nine tissue cultures showing

little cytopathic changes were suspended by EDTA and at 3 and 4 days after seeding, remarkable cytopathic changes were observed in two samples. The others did not show any change during an observation period of more than two weeks. From this result it is considered that in old tissue culture, the cell number is so large and multiplication of virus is so slow that cytopathic changes are hardly detectable. When cells in such a condition are seeded anew, the virus multiplication becomes active with restored cell metabolism. This presumption is supported by the fact described in the following article (Toyoshima *et al.*, 1959).

Though efforts had been made to isolate the virus with FL cells in our laboratory for a few months before the present experiment, no cytopathic change was observed even in the 1st inoculation. The present study shows that many specimens collected in the pandemic period of measles showed cytopathic changes in their earlier passages, while only one of specimens towards the end of the pandemic induced cytopathic changes in the 1st inoculation.

Antibody to Toyoshima strain was detected in convalescent sera and not in acute phase and uninfected human sera by the neutralization test, complement fixation test or by fluorescent antibody staining. The close interrelationship between the development of antibodies and the days of the disease was proved in the complement fixation test. Only one case was observed which had CF-antibody to Toyoshima strain without history of measles. We have the same opinion on this case as that described by Black (1959).

Immunologically and cytopathologically remarkable similarities were found between the Edmonston strain of measles virus and the Toyoshima strain.

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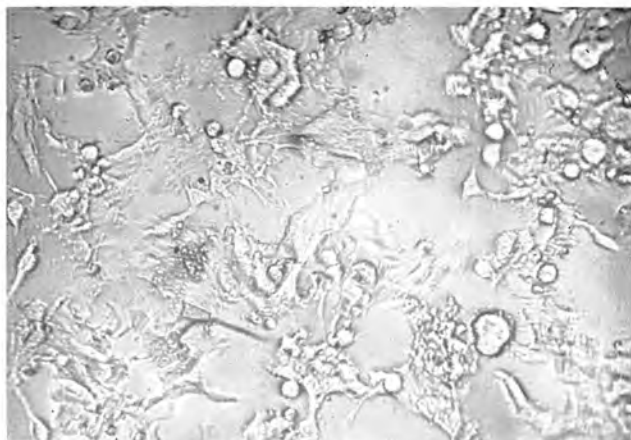


Fig. 2. CPC of FL cells infected with Toyoshima strain. H-E stain $\times 100$.

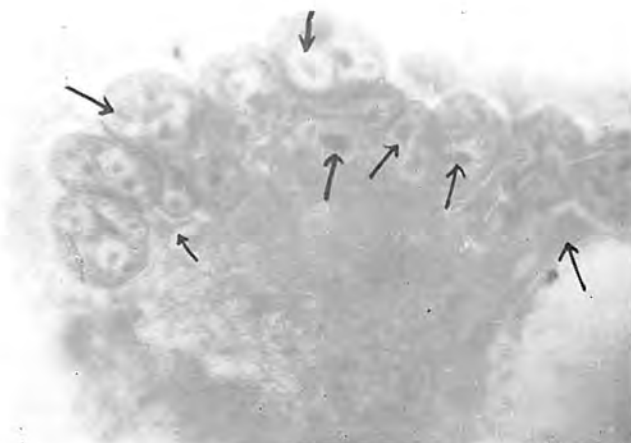


Fig. 3. A giant cell containing both intranuclear and cytoplasmic inclusion bodies in FL cell culture infected with Toyoshima strain. H-E stain. $\times 1,000$



Fig. 4. A cell bearing cytoplasmic eosinophilic masses in FL cell culture. Giemsa's stain. $\times 1,000$.

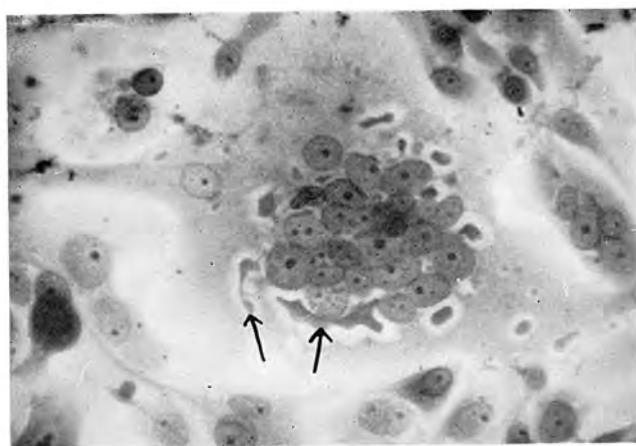


Fig. 5. Giant cells containing cytoplasmic inclusion bodies in monkey kidney tissue culture infected with Toyoshima strain. H-E stain $\times 40$.



Fig. 5. Monkey kidney cell cultures infected with Toyoshima strain. Indirect fluorescent antibody staining with anti-Edmonston human serum. $\times 200$. Distinct fluorescence was visible in nuclei of most cells and in cytoplasm of some cells.