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Virological Studies on Measles Virus IV. The Effect of Active and Inactivated Measles Virus on Cultured Cells

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SUMMARY

Active measles virus seems to possess two characters apart from its infectivity. The one is the ability to form giant cells in FL cell cultures. This character is relatively UV-stable and thermolabile. Both active and UV-inactivated virus produce giant cells when inoculated at high concentration into FL cell cultures. However heat-inactivated virus does not produce giant cells under the same conditions. The other character of this virus is its ability to alter susceptibility of the host cells to trypsin-citric acid treatment. This property of the virus is thermostable and UV-labile. The number of nuclei counted after trypsin-citric acid treatment, had decreased in infections by highly concentrated active or heat-treated virus, but not in infections by UV-inactivated virus.

INTRODUCTION

Giant cell formation is one of the characteristics of measles virus infections both in cell cultures (Enders and Peebles, 1954) and in experimental animals (Taniguchi *et al.*, 1954 a, b). Though a glutamine free medium has been reported by Reissig *et al.* (1956), to affect giant cell formation in cell cultures infected with measles virus, the mechanism of giant cell formation is unknown. On the other hand when a "giant cell" variant and a "strand-forming" virus were separated from the Edmonston strain by Seligman and Rapp, glutamine did not affect giant cell formation by the "giant cell" variant (1959). In our previous experiments (Toyoshima *et al.*, 1960 a), giant cells were produced sooner when the initial dose of virus was high and at earliest, appeared 10 hours after infection in unstained monolayer cultures. In an infection of high multiplicity, however, both the number of cells and the infective centers were less than those in an infection of moderate multiplicity. Many giant cells were obser-ed in a trypsin-dispersed preparations of highly infected cells though in an unstained monolayer cultures, giant cell formation was scarcely observable and only a lack of definite cell outline was noticed (Toyoshima *et al.*, 1960 a).

The effect of other active or inactivated viruses on cells has been reported by Okada *et al.* (1958) and Hanafusa (1960). The early changes in cells infected with measles virus was therefore investigated, and the effect of inactivated measles virus on cultured cells were studied.

MATERIALS AND METHODS

1. *Virus*

The Toyoshima strain of measles virus (Toyoshima *et al.*, 1959), extracted from infected cells by freezing-thawing, was sedimented by high speed centrifugation after removing the cell debris by low speed centrifugation (Toyoshima *et al.*, 1960b). The virus was then resuspended in maintenance medium (LE containing 3 per cent ox serum) at appropriate concentrations.

2. *Tissue culture cells*

FL cells grown in LE supplemented with 15 per cent ox serum and L cells in YLH (Hanks' balanced salt solution containing 0.5 per cent lactalbumin hydrolysate and 0.1 per cent yeast extract) with 5 per cent ox serum were used. Square tubes containing approximately 3×10^5 cells and one ml of medium were prepared for observation of cell changes. Cells from a single source were used in each experiment.

3. *Antimeasles serum*

The same monkey serum was used as for work described in the preceding reports (Toyoshima *et al.*, 1960a).

4. *Heat inactivation*

Sealed ampoules containing 1 ml of virus material were completely immersed in a water bath at 56°C. For inactivation, virus material was incubated for an appropriate time allowing an extra minute. Zero time material for inactivation experiments was therefore used after one minute's incubation at 56°C. After treatment ampoules were immediately cooled in an ice bath.

5. *Ultraviolet (UV) inactivation*

A 19 W germicidal lamp fitted with a reflector (Matsuda) was employed for inactivation experiments. Two ml of virus material in a 9 cm petri dish was irradiated with continuous shaking at a distance of 41 cm. UV-inactivated virus was usually prepared by irradiation for 3 minutes.

6. *Infectivity and CF antigenicity titrations*

The technique described in the preceding paper (Toyoshima *et al.*, 1960a) was employed for infectivity titrations except for the volume of the inoculm, where 0.1 ml was used rather than the 0.2 ml aliquot used in previous experiments. For CF antigenicity titrations, a modification of Kolmer's method (Toyoshima *et al.*, 1960b) was employed.

7. *Infection of cells*

Unless otherwise specified, cells were infected with 0.25 ml of virus material for 2 hours at 35°C. The virus was then discarded and the cells were washed once. Then 1 ml of maintenance medium was added to each square tube. Tubes were incubated at 35°C for appropriate times.

8. *Observation of cells*

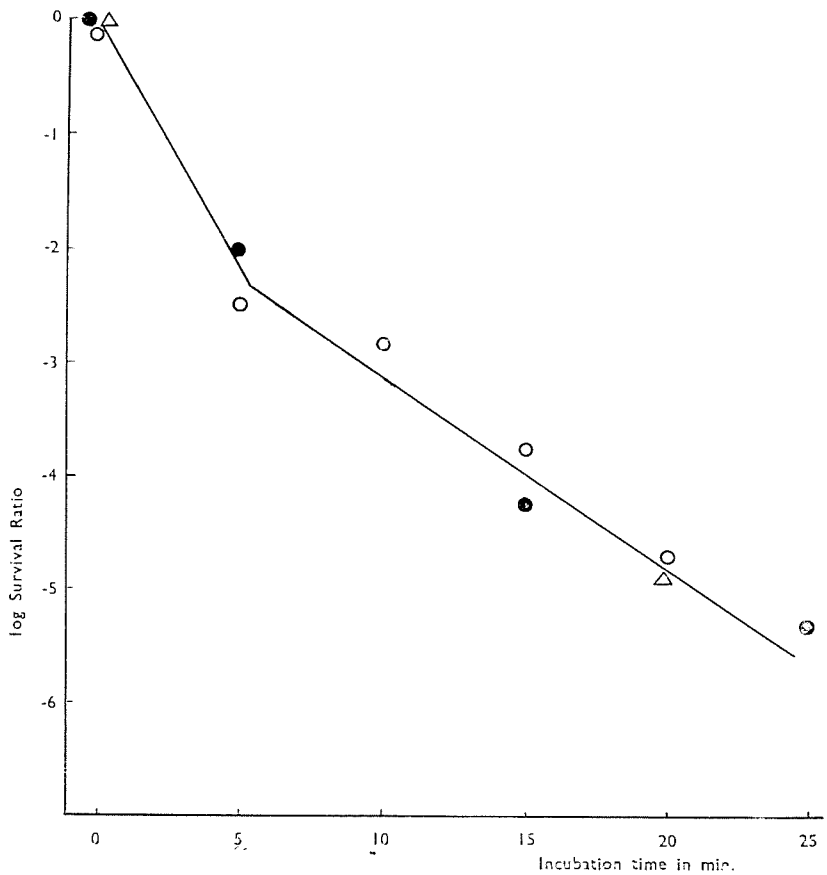
The medium was decanted and 0.5 ml of 0.25 per cent trypsin (Difco) in phosphate buffered saline was added. After 15 minutes' incubation at 35°C, cells were dispersed by pipetting and added to an equal volume of Türk's solution. Then the cell number was counted in a hemocytometer. One ml of trypsin-dispersed cells from the same tubes was centrifuged and the sediment was incubated with 0.1 M citric acid at 35°C with occasional shaking. The cells were centrifuged and the sediment was again treated with 1 ml of 0.1 M citric acid for 20 minutes. Then 1 ml of Türk's solution was added and the nuclei isolated were counted in a hemacytometer.

RESULTS

1. *Heat inactivation*

Heat inactivation experiments were performed at pH 7.0-8.0 because of the relative stability of the virus in this pH range (Black, 1959; Musser and Underwood, 1960). As shown in Fig. 1, virus was inactivated at a rate of about one log unit per 5

Fig. 1. Inactivation of Measles Virus at 56°C (pH 7~8)
Samples were incubated for 1 min. longer than the time indicated to allow the tubes equilibrate at the temperature.



minutes. This inactivation rate was compatible to that found by Black (1959). However, it was noted that the rate of inactivation changed after about 5 minutes and repeated treatment achieved the same results. Studies are now in progress on the problem of whether this difference was caused by experimental errors or by a variety in the thermal stability in this strain of virus.

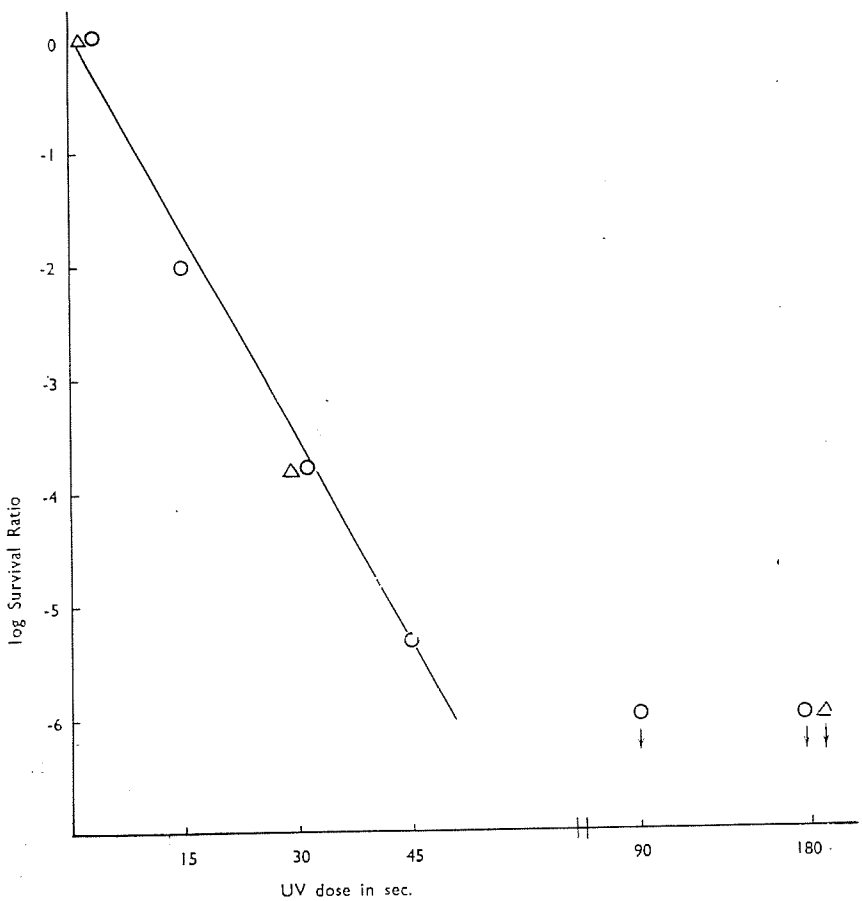
The CF antigenicity of virus materials was apparently not affected by incuba-

tion at 56°C for 20 minutes.

2. *UV inactivation,*

For this purpose, virus material was harvested from maintenance medium which did not contain phenol red. The virus having 10^6 $TCID_{50}$ per 0.1 ml was completely inactivated by irradiation for 90 seconds at a distance of 41 cm (Fig. 2), whereas the CF antigenicity remained unchanged even after irradiation for 3 minutes at a distance of 7.5 cm.

Fig. 2. Inactivation of Measles Virus by UV-irradiation*
*A 19 W. UV-germicidal lamp was employed for inactivation at a distance of 41 cm.



UV-inactivated virus absorbed considerable amounts of neutralizing antibody, though precise experiments on the quantitative analyses have not yet been made (Table 1).

Table 1. Absorption of Neutralizing Antibody* by UV-inactivated Measles Virus

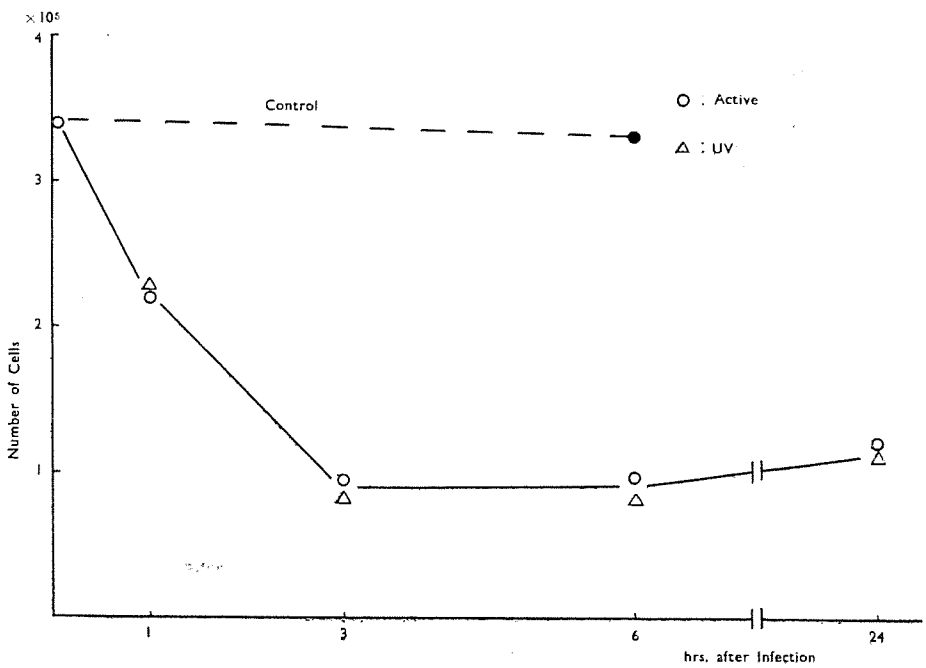
Inacti-vated \ Serum dil. / Active		8	16	32	64	128
UV	$TCID_{50}$/tube 21		0/4	0/4	0/4	3/4
Control	21	4/4	4/4	4/4	4/4	4/4

Results are expressed as CP positive per total number of tubes.
UV : UV-inactivated virus which had 10^6 $TCID_{50}$ before inactivation.
Control : Maintenance medium
* Serum was incubated with inactivated virus at 37°C for 30 min. and then at 4°C overnight. Then the mixture was mixed with active virus and incubated for 1 hour at room temperature before inoculation.

3. *Giant cell formation in high multiplicity-infections*,

a) Time for giant cell formation: The decrease in cell number was taken as an index of cell fusion because it was found the most reliable indicator. Small fused cells could be observed in FL cell cultures one hour after infection with active or UV-inac-

Fig. 3. Number of Cells after Infection



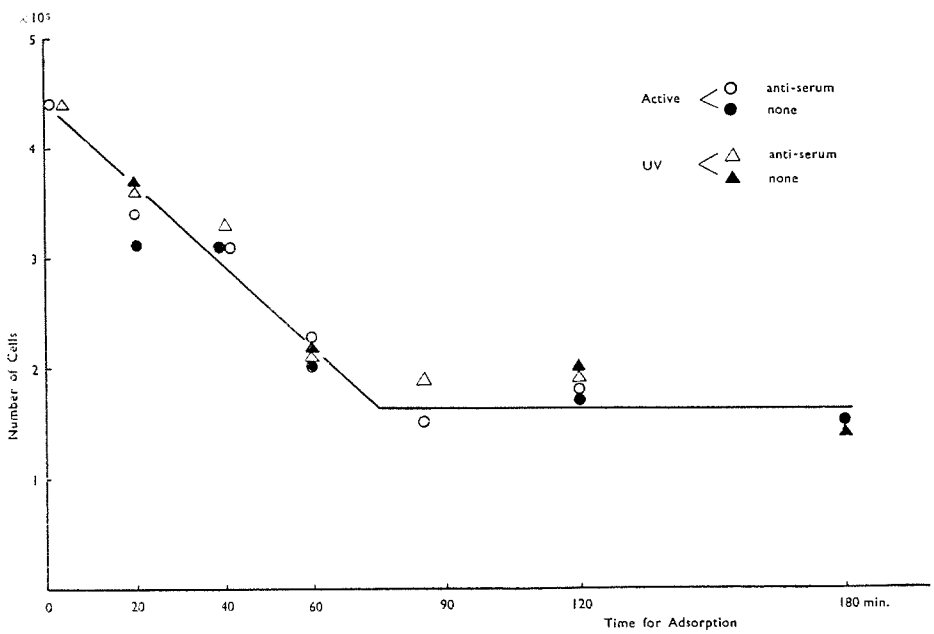
tivated virus (Figs. 7, 8). The fusion of cells had increased greatly by 3 hours after infection (Figs. 9-12) but thereafter practically ceased (Fig. 3). However, at the same concentration, virus which had been partially inactivated by treatment at 56°C for 20 minutes, did not produced giant cells (Fig. 13). Giant cells produced by infection

with UV-inactivated virus degenerated gradually within several days but they produced neither infective progeny nor a detectable amount of CF antigen.

In L cell cultures, which are not suitable for growth of measles virus, neither active nor inactivated viruses even at high concentration produced giant cells.

b) Time for adsorption and grade of giant cell formation: FL cells were incubated with virus material at 35°C. At appropriate intervals 4 square tubes were washed once. Two were incubated with 1 ml of 10 per cent antimeasles monkey serum in LE and the other two were incubated with 1 ml of 10 per cent control serum in LE at 35°C for 1 hour. The medium was discarded and after washing the tubes was replaced by 1 ml of maintenance medium. Cells were counted 6 hours after infection, since this period had been shown to be sufficient for giant cell formation (Fig. 3). Fig. 4 shows the results of infection with active and UV-inactivated virus. An adsorption

Fig. 4. Number of Cells and Time for Adsorption of Virus

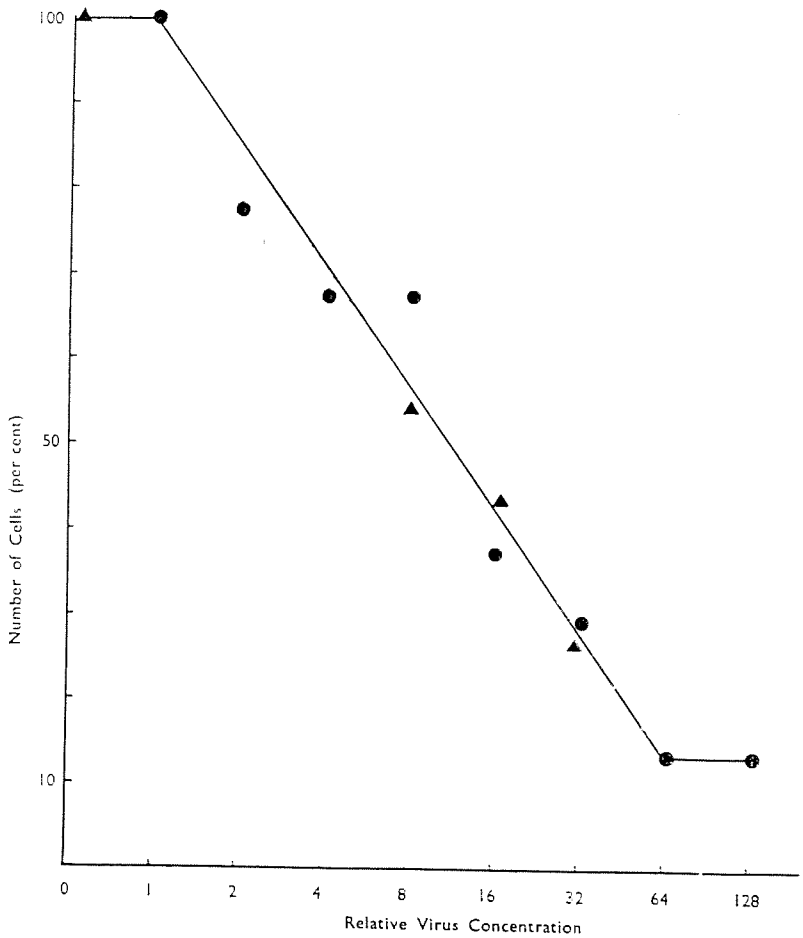


The number of cells was determined 6 hours after infection.
Zero time : Virus material was mixed with $\frac{1}{10}$ volume of antiserum and incubated for an infection period of 2 hours.

time of more than 90 minutes had little effect on giant cell formation and antiserum did not affect after adsorption of the agent onto the cells. No difference could be observed between the effect of active and UV-inactivated virus.

c) The effect of the concentration of imput virus: Two hours after infection, the incubation fluid was replaced by maintenance medium. The cell number was determined 6 hours after inoculation. Fig. 5 shows that within a certain range a linear re-

Fig. 5. Number of Cells and Concentration of Imput Virus



The number of cells wos determined 6 hours after infection.

lationship was observed between the number of cells and concentration of imput virus. Giant cells were scarcely formed at the lowest concentration of virus and the number of cells in this preparation was compatible with that in the untreated cultures. At the highest concentration of virus used, degenerated cells were observed and giant cells were smaller than those formed at the second highest concentration used. The lowest virus concentration at which giant cells were formed under these conditions was about 10^6 $TCID_{50}$ per 0.1 ml. The giant cell forming ability of this virus was not much affected by increasing the UV-dose up to 10 times more than that required for complete inactivation of the virus.

d) The effect of super-infection with a high concentration of virus: Pairs of square tubes were inoculated with various concentrations of virus. Two hours after-

wards the fluid was replaced by a higher concentration of virus. 6 hours after the first inoculation, the number of cells were determined. Though giant cells were formed to some extent by the initial inoculum, the number of cells decreased more evidently by further addition of more concentrated virus (Table 2). Prior infection with smaller

Table 2. The Effect of Prior Infection at a Low Concentration on Giant Cell Formation by a Second Infection of Higer Concentration.

Relative virus Concentration		1st Virus infection								
		0	1	2	4		8		16	
			A	A	A	UV	A	UV	A	UV
2nd Virus infection	0	30	23	20	20	NT	11	NT	9.0	NT
	32	6.7	6.1	6.0	5.9	5.2	5.8	5.6	6.4	5.8

Results are expressed as the number of cells ($\times 10^6$).
A: Active virus
UV: UV-inactivated virus
NT: Not tested

inoculums did not seem to affect giant cell formation by a second infection with a higher concentration of virus.

4. *The change in susceptibility of infected cells to trypsin-citric acid treatment,*

Infection with heat-inactivated virus did not cause giant cell formation, but many

Table 3. Changes in Susceptibility of Infected Cells to Trypsin-Citric Acid Treatment.

a		No Virus	Active	UV-irradiated		Heated at 56°C (min.)			
				41 cm 3 min.	7.5 cm 4 min.	5	10	15	20
Cell	Number (10^3)	135	28	40	85	147	136	117	120
	% Control	100	21	30	63	109	100	87	89
Nucl.	Number (10^3)	130	60	103	114	58	87	85	90
	% Control	100	46	78	82	45	67	65	69
Nucl./Cell		0.96	2.1	2.6	1.3	0.39	0.64	0.74	0.75

b		No Virus	Active	UV-irradiated at 41 cm (min.)			Heated at 56°C (min.)			
				2	3	4	5	10	15	20
Cell	Number (10^3)	342	68	63	73	65	304	325	337	325
	% Control	100	20	18	21	19	89	95	99	95
Nucl.	Number (10^3)	327	190	247	263	260	196	201	202	204
	% Control	100	58	76	80	80	60	61	62	62
Nucl./Cell		0.96	2.9	3.9	3.6	4.0	0.64	0.64	0.65	0.74

In (a) each figure represents the average of 4 tubes and in (b) of 3 tubes.

degenerating cells could be seen in trypsin-dispersed preparations (Fig. 13). These degenerating cells were also present in cell cultures infected with active virus, but they were few in cultures infected with UV-inactivated virus. Cells infected with active or inactivated virus were dispersed by trypsin treatment 6 hours after infection and number of cells was determined. Another part of same sample was used to count the nuclei after citric acid treatment.

Because of the complexity of the process used for isolation of nuclei, the results are not fully reliable, especially in the first experiment in which the original number of cells were unexpectedly low. But from the results of 2 experiments (Table 3a and b), the number of cells seemed to decrease greatly after infection with both active and UV-inactivated virus but not after infection with heat-inactivated virus. On the other hand, the number of nuclei decreased after infection with active or heat-inactivated virus, and not greatly after infection with UV-inactivated virus. The reduction in the number of nuclei caused by active virus was comparable to that caused by virus which had been heated for 5 minutes and which had consequently lost more than 90 per cent of its infectivity.

DISCUSSION

The strikingly early appearance of giant cells in preceding experiments (Toyoshima *et al.*, 1960a) led us to study the early changes in infected cells. The effect of inactivated virus was also studied because giant cell formation and other effects of inactivated HVJ and vaccinia had been reported by Okada *et al.* (1957, 1958) and Hanafusa (1960).

Active and UV-inactivated measles virus could produce giant cells in FL cultures when inoculated at a concentration of more than 10^6 $TCID_{50}$ per 0.1 ml. More giant cells were formed when the concentration of imput virus was high, and the appearance of giant cells was nearly maximal 3 hours after infection. These changes were inhibited by mixing the virus with antimeasles serum beforehand, but antiserum did not affect the change after adsorption of virus onto the cells. On these points, no difference could be observed between active and UV-inactivated virus, whereas heat-inactivated measles virus could not produce giant cells. The giant cells formed by measles virus are not merely due to agglutination of the cells as reported by Mayyasi who worked with vaccinia virus (1959). Multiplicity reactivation was not observed even with the high multiplicity of infection of UV-inactivated virus which produced many giant cells in FL cell cultures. Both active and UV-inactivated virus failed to produce giant cells in L cell cultures. This cell line seems to have a very low susceptibility for this virus. Whether this is related to the absence of giant cell formation in L cells after infection is not certain at present.

Heat-inactivated virus could not produce giant cells either in FL or in L cell cultures, but seemed to have a cytotoxic effect so that the susceptibility of cells to trypsin and citric acid-treatment was altered. Active virus also seemed to have this

character while UV-inactivated virus did not.

These results suggest the following conclusions: Active measles virus has two different characters apart from its infectivity. These are the ability to cause giant cell formation and the ability to alter the susceptibility of host cells to trypsin and citric acid-treatment. One of these characters, the ability to cause giant cell formation, is relatively stable to UV-irradiation but is thermolabile. The other character is thermostable and UV-labile.

Interference in giant cell formation was not detected either by active or by UV-inactivated measles virus under the conditions employed. Giant cell formation by this virus differ from that by HVJ virus in that there is an absence of interference. For giant cell formation by HVJ virus, a high concentration of cells and shaking cultures were required (Okada, 1958). In our experiments, giant cells were formed in stationary cultures containing about 10^5 cells per 1 ml. These differences, however, seems to be of little importance in view of the great difference between suspended cell cultures and monolayered cells.

Though the toxic substance was separated from the infective particles of adenovirus, the agents of measles virus responsible for these cell changes could not be separated by differential centrifugation. Studies on the possibility of fractional separation using column chromatography are now in progress in our laboratory.

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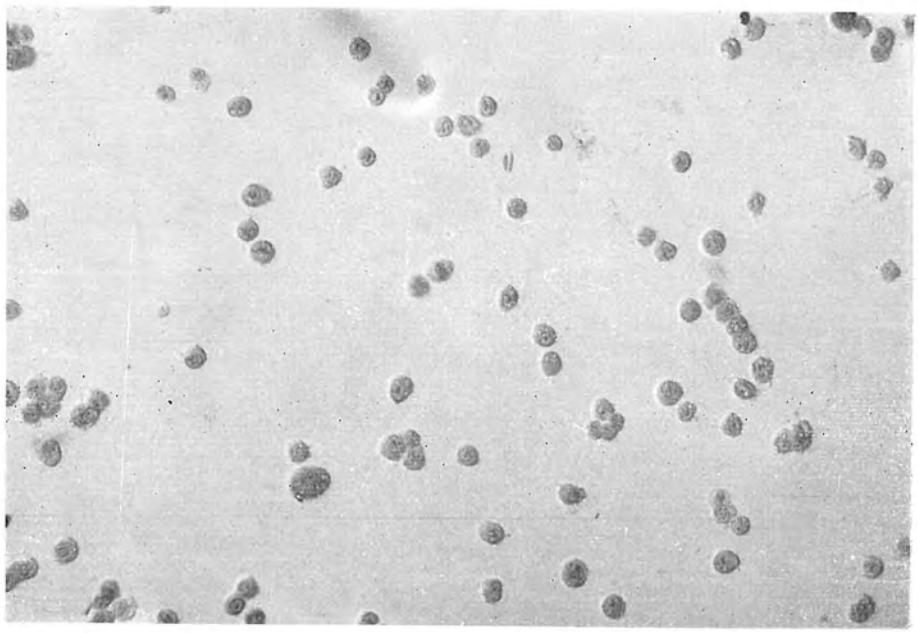

Fig. 6. Uninfected FL cells dispersed by trypsin-treatment (t-t). ×100

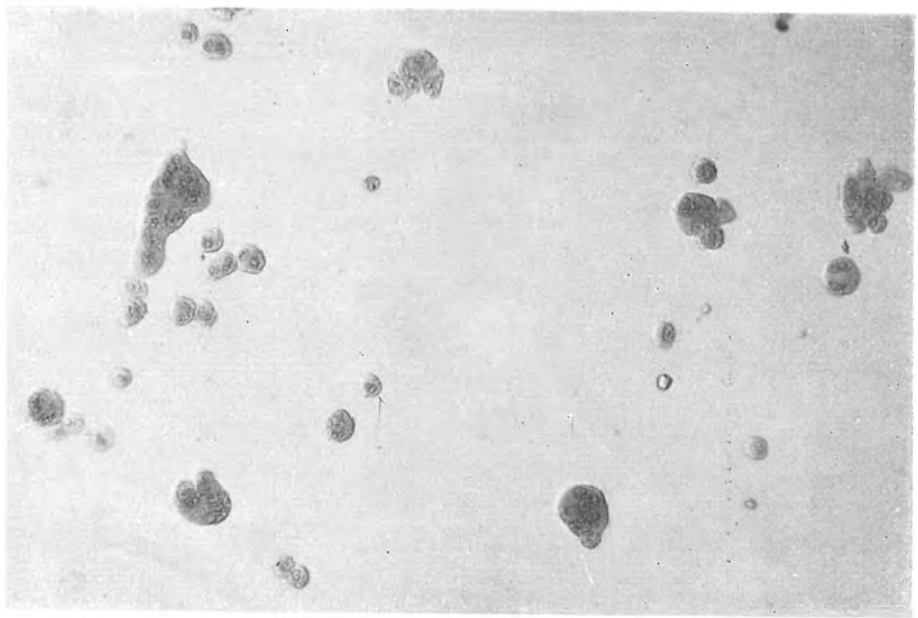

Fig. 7. One hour after infection with active virus. FL cells dispersed by t-t. ×100

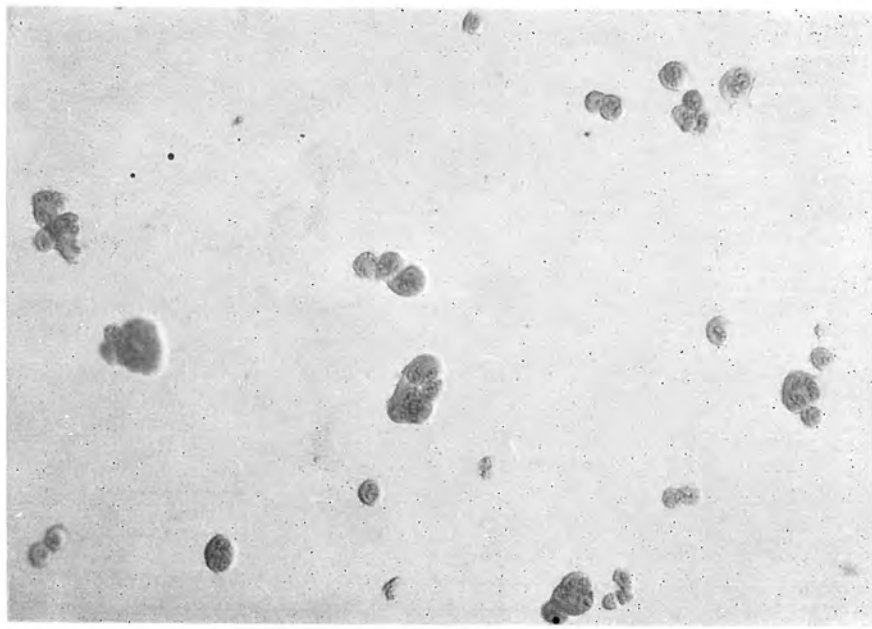

Fig. 8. One hour after infection with UV-inactivated virus. FL cells dispersed by t-t. ×100

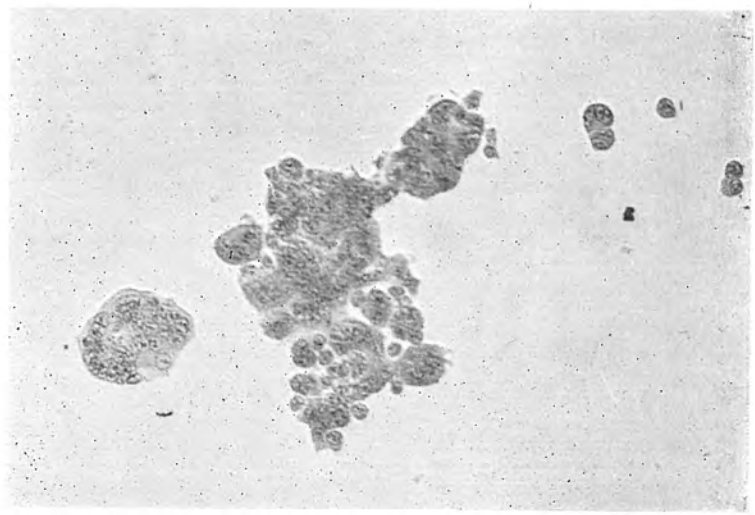

Fig. 9. 6 hours after infection with active virus. FL cells dispersed by t-t. ×100

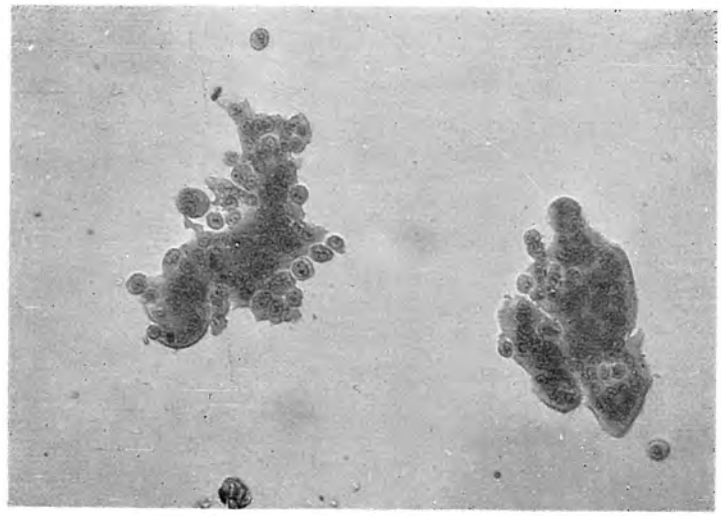

Fig. 10. 6 hours after infection with UV-inactivated virus. FL cells dispersed by t-t. ×100

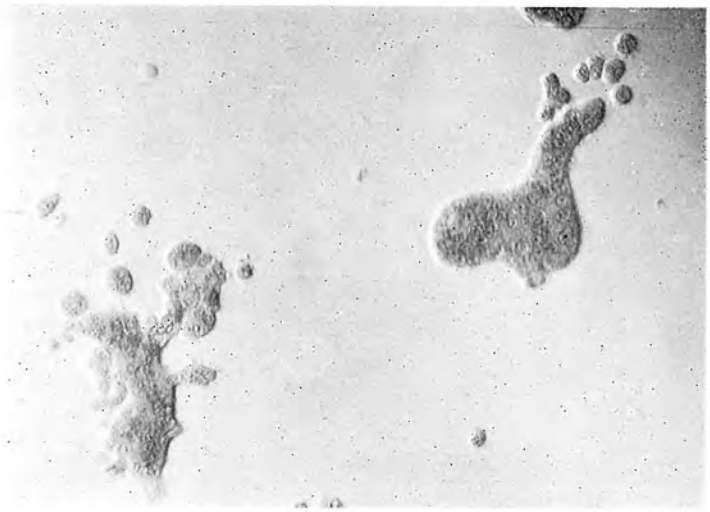

Fig. 11. 24 hours after infection with UV-inactivated virus dispersed by t-t. ×100

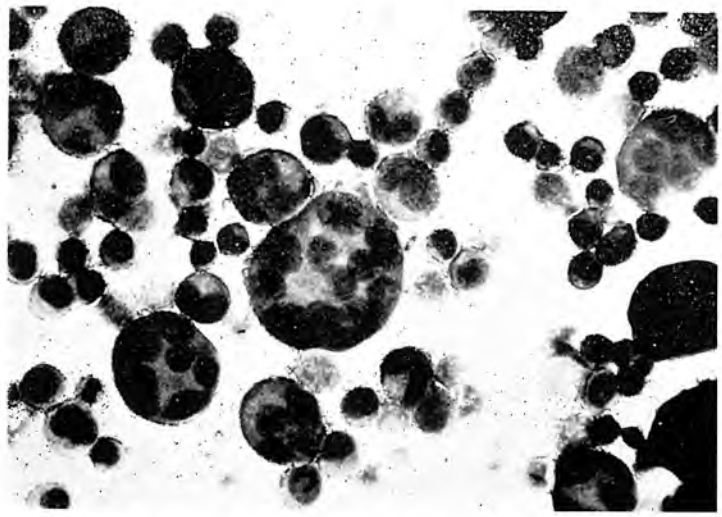

Fig. 12. 6 hours after infection with UV-inctivated virus. Smear preparation of FL cells sedimented by centrifugation after t-t. Giemsa staining. ×200

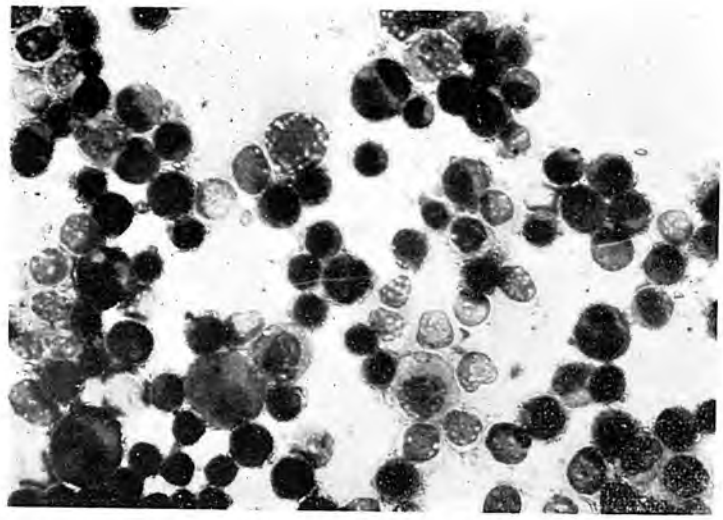

Fig. 13. 6 hours after infection with heat-inactivated virus. The same procedure used as for Fig. 12. ×200