BIKEN'S JOURNAL 3, 191-199 (1960)

Killing of L- Cells by Heat- and UV- Inactivated Vaccinia Virus

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(Received for publication, June 20, 1960)

SUMMARY

The cytopathogenic effect of heat- and UV- inactivated vaccinia virus on L cells and their ability to kill the host cells are described. These two inactivated forms of virus cannot multiply, but they are still lethal to host cells in their respective manners. Heated virus causes only a slow shrinkage of cells. Multiple infection with heated virus has been shown to be necessary for death of the cells. The cytopathic changes induced by UV-irradiated virus seems to correspond to those caused by the virus especially at an early stage of infection. The cell killing capacity is rather heat labile in UV irradiated virus, while in heated virus it is UV sensitive.

INTRODUCTION

Our previous studies on various biological properties of heat inactivated vaccinia virus have shown that the inactivated virus was reactivated when there was mixed infection with active heterologous pox-viruses. The inactivated form could also interfere with the multiplication of active homologous virus, though its infectivity was completely lost (Hanafusa *et al.*, 1959 a, 1959 b, 1960).

While studying the reactivation phenomenon, it was found that when high titer heat-inactivated vaccinia IHD (with an infection multiplicity of more than 4) was inoculated into an L cell culture, cellular degeneration appeared without viral replication and most of the cells had died 15 hours after inoculation. But the cytopathic changes caused by heated virus were quite different from those caused by the active virus in that the rounding and agglutination of the cells, which is always noticeable at an early stage of infection with the active virus, did not occur. On the other hand, UV-inactivated virus had a different kind of cytopathogenicity. The cytopathic changes induced by irradiated virus, which can be seen several hours after infection, closely resemble those caused by active virus throughout the entire cycle of infection.

This paper describes the cytopathic changes* and death of the host cell following infection with heat- and UV-inactivated virus, and some properties of these cell-killing agents.

* Since all of active virus, heat- and UV- inactivated ones finally kill the host cells, the term "cell-killing" is used in this report. But sometimes the term "cytopathogenicity" is also used, when cytopathic change of host cells is particularly considered.

MATERIALS AND METHODS

1. *Cells*

Earle's L cells were used. The cells were grown in monolayer cultures in Hanks' saline containing 0.5% lactalbumin hydrolysate, 0.1% yeast extract (La-Ye) and 5% calf serum.

2. *Virus*

The IHD strain of vaccinia virus grown in L cells was used throughout this study. Infected cells suspended in the medium were disrupted by sonic vibration at 10,000 cycles per second for 10 minutes in a Kubota 200-watt 10 KC magnetostrictive oscillator. Sonorated virus suspensions were treated with fluorocarbon ("Daifron S3", Osaka Kinzoku Co., Japan) according to the method of Epstein (Epstein, 1958), and finally purified by one cycle of differential centrifugation.

3. *Assay procedure*

The titration of infectivity was carried out in L cell cultures by plaque assay, as described in an earlier report (Hanafusa *et al.*, 1959a). The capacity to be reactivated of heated virus was determined by the plaque method as described in the preceding paper (Hanafusa, 1960).

4. *Inactivated virus*

Heat treatment: Vaccinia virus of $0.5 \sim 2 \times 10^8$ PFU*/ml was heated in an ampoule at 56°C for one hour.

Ultraviolet light (UV) treatment: Ultraviolet irradiation was performed with a 15 watt germicidal tube (Toshiba Electrics). The virus stock (5×10^7 PFU/ml) to be inactivated was exposed to UV light for 2 minutes in 2 ml aliquots in a petridish (87 mm I.D.) at a distance of 40 cm from the lamp. Samples were rocked gently during irradiation. Judging from the UV-inactivation curve shown in Fig. 1, most of the infectivity was lost under the above con-

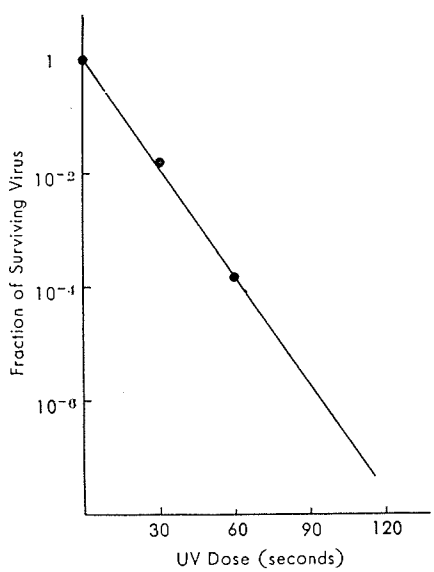

Fig. 1. UV inactivation of infectivity of vaccinia virus.

* PFU = Plaque forming unit

ditions. It was shown that neither the heat nor the UV inactivated virus preparations prepared as above contained surviving infectious virus.

5. *Inoculation of samples*

All experiments were carried out on monolayers of L cells containing approximately 5×10^6 cells, grown in 200 ml prescription bottles. One ml of each sample was adsorbed on the cell layer at 37°C for 2 hours. Then the cells were washed with 4 ml of Hanks' saline to remove unadsorbed material and overlaid with 8 ml of fresh medium.

In these experiments the multiplicity of heated virus was calculated from the multiplicity of active virus used for heat inactivation assuming that heat treated virus was adsorbed at the same rate, since the estimation of adsorption of heated virus was very difficult. From the previous studies on the properties of heated vaccinia (Hanafusa, 1960), this assumption was considered to be valid.

6. *Vital staining of cells*

Viability of cells was estimated by staining them with methylene blue. After the culture medium had been removed, the cells were harvested, centrifuged at 1000 rpm for 5 minutes and resuspended in 1 ml of 0.01 % methylene blue in phosphate buffered saline. Within a few minutes an aliquot of this suspension was examined in a haemocytometer. The number of dead cells staining with methylene blue and of healthy ones, which did not stain, were counted. A minimum of 1000 cells was counted in every sample. There was usually about 5~8 per cent of blue-stained cells in normal untreated L cells. These may have been killed during harvesting and centrifugation. Cells heated at 56°C for 30 minutes or treated with 0.01 M KCN solution for 5 minutes were completely stained by this method.

RESULTS

1. *Cell killing by heated virus*

Vaccinia virus multiplies in L cells, inducing a remarkable degeneration in the host cells. In the first 2~3 hours after infection the cells begin to round up and become separated from each other. Polynucleated giant cells are formed in considerable numbers through agglutination. Finally about 24 hours or later after infection, the cells lyse producing more than one hundred virus progeny.

Heat inactivated virus causes no morphological changes in the host cells when multiplicity of infection was about 1 (Hanafusa, 1960). But when large doses of heated virus were inoculated, most of the cells shrank and the monolayer sheet was destroyed 24 hours after inoculation (Fig. 2). Finally the cells were detached from the glass surface and lysed. This process of killing the cells was not accompanied by any viral growth. Thus, in the cultures inoculated with the heated virus of 2~4 multiplicity, partial degeneration was found at 24 hours, but later the degenerated lesions disappeared as the remaining healthy cells grew. As cell killing by heated virus took some time, damaged cells appeared only after 10 hours. The initial response of the cells to infection by active virus was absent in this case and neither inclusion bodies nor viral antigen were observed by the giemsa staining or fluorescent antibody technique.

2. *Inactivated virus particles as cell killing agents*

The first question which arises from these findings is whether this phenomenon is caused by heated virus itself or by other cellular constituents and other particles produced in infected cells. Although virus preparations purified with fluoro-

carbon were used in these experiments, the presence of a very small amount of some other substance in the preparation could not be excluded. In order to solve this qtestion various experiments were performed.

Uninfected L cells were disrupted by sonic oscillation and centrifuged under the same conditions as used to prepare the virus stocks. Neither the supernatant fluids from each batch of disrupted L cells nor a sample of each heated at 56°C for 1 hour caused any cellular degeneration. The cell killing ability of a heated virus preparation was completely abolished if the heated virus is neutralized with anti-vaccinia serum prior to being reacted with the cells. Further, by centrifugation of heated virus at 34,850 *g* for 30 minutes, all of the cell killing activity was precipitated and the supernatant fluid had no killing activity. These experiments suggest that heated virus particles act as cell killing agents.

3. *Quantitative study of cell killing*

As mentioned above, the extent of cell killing varied with the multiplicity of the heated virus. In order to investigate this problem quantitatively, the fraction of surviving cells was determined from the viable count.

The relationship between the fraction of surviving cells and the time after inoculation with a heated virus, with a multiplicity of 10, is shown in Fig. 3. The fraction of surviving cells dropped sharply after 10~15 hours and then decreased gradually until 48 hours after inoculation. The results were consistent with microscopic observations on cell cultures and all the shrunken cells stained with methylene blue.

However, the viable counting of cells was carried out at 24 hours after inoculation with heated virus to determine the cell killing power, because the effect of growth of unattacked cells on the results could not be neglected in samples of lower multiplicity.

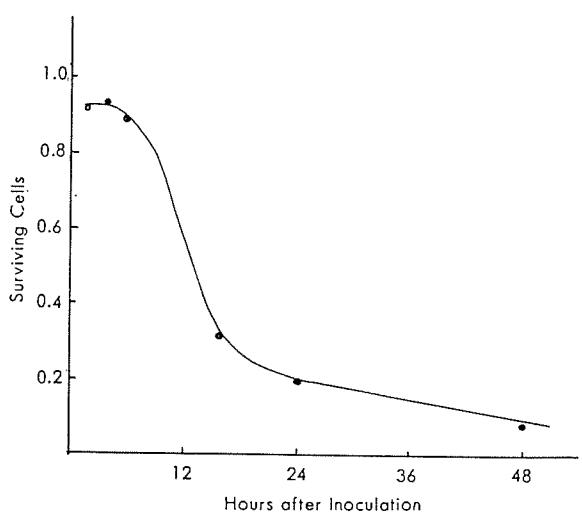

Fig. 3. Time course of death of L cells by heated vaccinia virus.

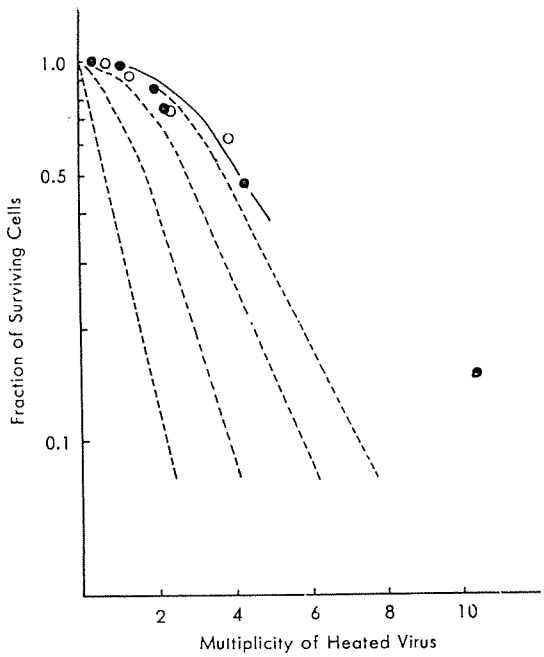

Fig. 4. Relationship between surviving fraction of L cells and multiplicity of heated vaccinia virus. Viability of the cells was estimated 24 hours after inoculation. The theoretical plots for 1～4 particle mechanisms of kill are indicated.

L cell cultures exposed to different multiplicities of heated virus, were stained with methylene blue at 24 hours after inoculation and the surviving cells were counted. The fraction of survivors varied with the multiplicity of the heated virus as shown in Fig. 4, where the theoretical plots* for one to four particle mechanisms of killing are indicated. Evidently the experimental data fit a 4-particle curve. The exact number of particles required to kill a cell could not be determined, for the multiplicity of the heated virus could only be assumed to be that of active virus. However these results indicate that the cell killing mechanism with heated virus is of a multiple hit type and only cells attacked by more than two particles are destroyed. The above facts are compatible with the facts that no cytopathic change was observed with heated virus with a multiplicity of about 1.

* The theoretical curves have been derived from the Poisson distribution, $P(r) = m^r \cdot e^{-m}/r!$, where P (r) is the probability of the cells in a given population being attacked by r heated virus particle when the average multiplicity of infection is m. The fraction of cells escaping killing can be calculated by the following equations:

1 particle curve ..... $P(0) = e^{-m}$
2 particle curve ..... $P(0)+P(1) = e^{-m}(1+m)$
3 particle curve ..... $P(0)+P(1)+P(2) = e^{-m}(1+m+m^2/2)$
4 particle curve ..... $P(0)+P(1)+P(2)+P(3) = e^{-m}(1+m+m^2/2+m^3/6)$

4. *Some properties of the cell killing agent*

The effects of antibody, trypsin, heat and UV-irradiation on this capacity of heated virus were examined under the conditions used in studies on other biological activities of heated vaccinia (Hanafusa, 1960).

As described in section 3, the cell killing capacity was completely abolished by reaction with antiserum before inoculation of heated virus into the cell culture. However, when antiserum was added to cells which had been exposed to heated virus for 2 hours, the cell killing took place as usual after 20 hours. This capacity was not affected by treatment with 50 μg/ml of trypsin. UV-irradiation, which caused a decrease in infectivity of active virus of 10^{-8}, inactivated the cell killing power of heated virus. These properties of the cell killing capacity are very like other biological capacities of heated virus. However the cell killing capacity was more heat stable than other capacities as shown in Fig. 5. Therefore, cell killing may be caused by a heat-stable and UV-sensitive fraction of heated virus.

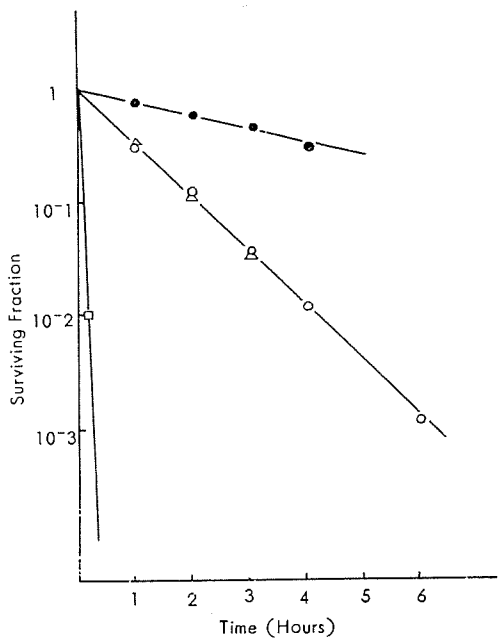

Fig. 5. Thermal inactivation (56°C) of some biological capacities of heat inactivated vaccinia virus. Capacity to be reactivated (○), capacity of interference (△), cell killing capacity (●). The inactivation curve of infectivity (□) of active vaccinia is also indicated.

5. *Reactivation and cell killing*

Even in the cells which would have been killed by multiple infection of heated virus, reactivation of heated virus occurred by superinfection with active ectromelia. Monolayers of L cells were exposed to a mixture of active ectromelia (2×10^4 PFU) and heated vaccinia sufficient to cause death of all the cells. After

2 hours the inoculum was removed and then the cells were washed several times with Hanks' saline. A uniform suspension of these cells was prepared by scraping and aspirating with a pipette, and appropriate dilutions of cell suspension were plated on the monolayer of L cells. The number of reactivated vaccinia on the plate coincided with the expected value assuming that reactivated virus was released from all cells infected with ectromelia. However, when the active ectromelia was superinfected at 24 hours after inoculation of heated virus of high multiplicity, no reactivated virus could be detected using the same experimental procedures. This indicates that cells, in which the cell killing reaction has proceeded to a certain extent, lose the capacity to support reactivation.

6. *Cytopathic effects of UV-irradiated virus*

Virus particles inactivated by UV-irradiation also caused death of host cells, but the cytopathic changes were very different. The various changes induced by irradiated virus were indistinguishable from those caused by active virus except that no morphological signs of viral growth, i. e. formation of inclusion bodies and viral antigen, were found. UV-irradiated virus caused rounding of the cells and formation of giant cells to the same extent as did active virus (Fig. 6). Multiple infection of UV-treated virus was also lethal to the cells. On staining with methylene blue it was found that the surviving fraction of cells after exposure to UV-irradiated virus was about 0.5, 24 hours after infection. This result is identical with the fraction of surviving cells after active virus infection. However, the cell counts were unreliable, as many giant cells were present. It is interesting that at this stage some giant cells stained with methylene blue and some did not. However, when a diluted suspension was used, some cytopathic changes were observed one day after infection but these disappeared later in the same way as the changes caused by diluted heated virus.

The cell killing activity of UV-irradiated virus was abolished by treatment of the virus with antiserum prior to its inoculation into the cell cultures though it could not be destroyed by trypsin. The activity was completely lost on heating the preparation at 56°C for 1 hour. The results show that the substance which causes the early cytopathic changes may be UV-resistant and heat-sensitive in contrast with the cell killing agent of heated virus.

DISCUSSION

Many kinds of animal viruses cause various cytopathic changes in susceptible cells. These have been regarded as evidence for viral multiplication. Further, there are a wide variety of cytopathogenic effects in cells infected with a single kind of virus. A cytotoxic agent separable from the infectious particles was demonstrated with adenovirus Type 3 and Type 5, (Pereira, 1958; Everett and Ginsberg, 1958). It is a protein like substance produced in infected cells, and its cytotoxic effects correspond to those of an early stage of virus infection. UV-irradiated virus also possesses the same cytopathogenicity (Pereira and Kelly, 1957; Levy *et al.*, 1957). The cell killing power of active New Castle disease virus has been investigated in detail by Marcus and Puck (1958). They showed that infection of one

virus particle is sufficient to cause death of the cell and that this cell killing agent is heat labile and UV sensitive. It is interesting that the number of cell killing particles exceeds that of plaque forming particles in NDV-vaccine strain (Marcus, 1959). Recently the agglutination of L cells by the IHD strain of vaccinia was investigated by Mayyasi *et al.* (1959). However their work was mainly on the agglutination caused by active virus and the utilization of this phenomenon for titration of virus and its neutralizing antibody.

The experiments presented here show that both the inactivated forms of vaccinia virus can induce death of the host cells, but that their mode of action is quite different. Studies on the cytopatogenicity of UV-irradiated virus suggest that the early cytopathic change caused by active virus may be due to a UV-resistant protein like substance and may be independent of the ability of the virus to reproduce. On the other hand, cell killing by heated virus seems to be correlated with the metabolism of the host cells, for it takes longer than that by active virus and causes only the shrinkage of the cells attacked. In this respect, the fact that the cell killing capacity of heated virus is UV-sensitive, is very suggestive. This activity may be the result of some unknown function of viral nucleic acids and might be correlated with the interference by heated virus. The disturbance of normal metabolic processes of host cells caused by heated virus may result in the establishment of interference and death of the cell. In addition, the reactivation of heated vaccinia virus by superinfection with active ectromelia took place under conditions where cell death might have occurred. Half the cells infected with active virus did not stain with methylene blue 24 hours after infection. Presumably the cells in which viruses are growing are living. Therefore cell death caused by heated virus may be due to a different reaction from that of active virus or to some defect in the normal process of viral growth. These possibilities are rather similar to cell killing by ghosts of T_2 bacteriophage. The killing action of ghosts is prevented if the cells are infected with bacteriophage (Garen and Kozloff, 1959). However, the killing phenomenon with heated vaccinia may be more complicate as multiple infection is necessary for death of the cell. Further studies on other biological aspects of inactivated viruses may throw light on the interaction between host cells and inactivated viruses.

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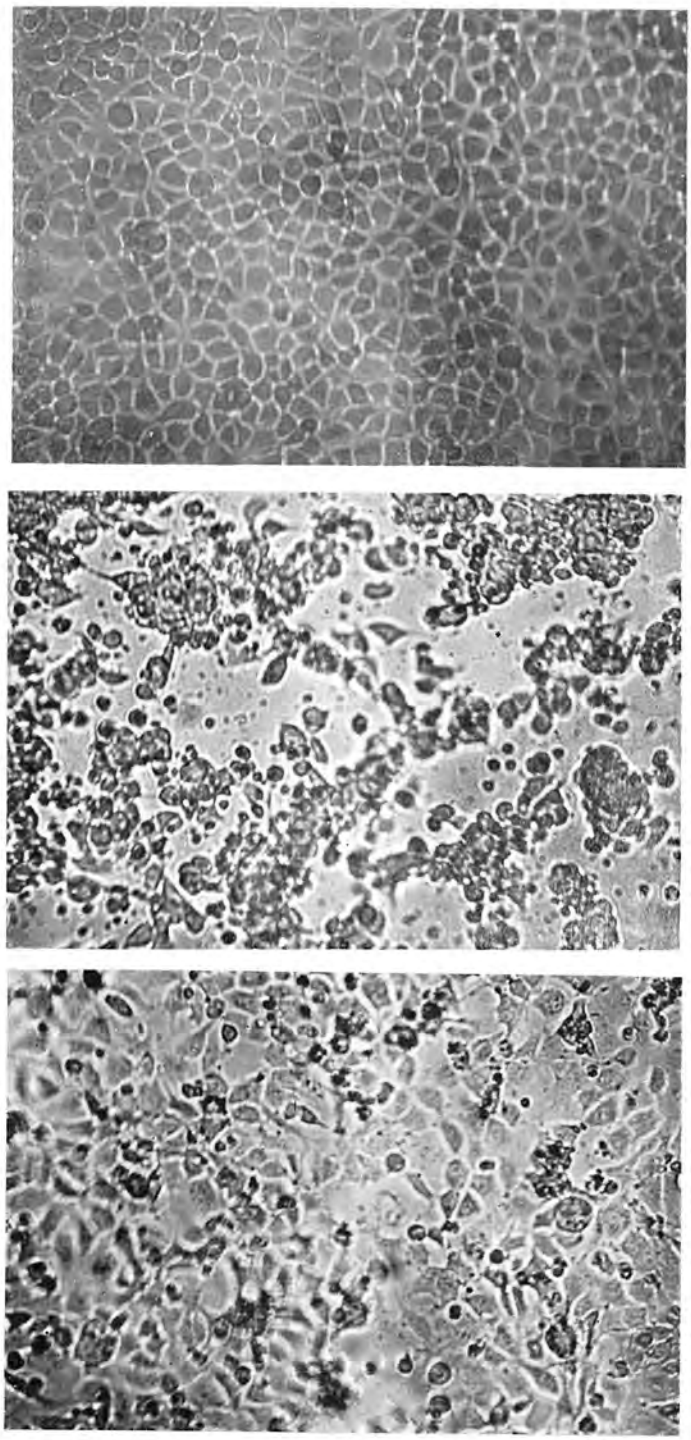

Fig. 2. L cell culture; (a) normal; 24 hours after inoculation with heated vaccinia IHD at (b) multiplicity 10 and (c) multiplicity 5. Magnification: ×105

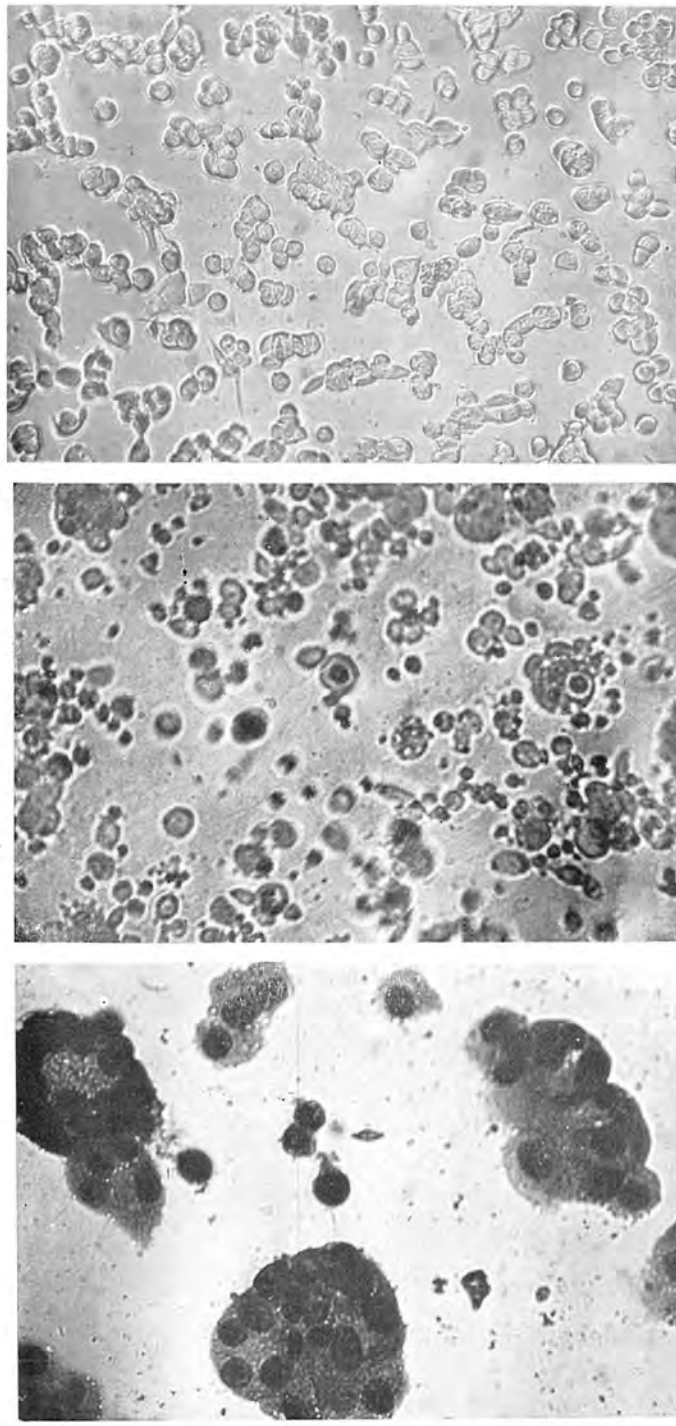

Fig. 6. L cell culture (a) 6.5 hours and (b) 24 hours after inoculation with UV irradiated vaccinia (Multiplicity 5). Magnification : ×105 ; (c) Giant cells in this culture (fixed with methanol and stained with Giemsa solution). Magnification : ×210