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Studies on the Growth of a Newly Isolated* ** Herpes Simplex Virus *in vitro*

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SUMMARY

1. On passage of a newly isolated herpes simplex virus (Miyama strain) on FL monolayers two substrains were obtained from the parent virus population. One causes syncytial formation of infected cells and the other does not.

2. These two types of virus also showed differences in their growth curves.

The giant cell-producing virus (+GC strain) caused early destruction of FL cells, while cells infected with the virus which did not form giant cells (—GC strain), only became rounded and remained attached to the wall of the glass culture bottle for several days. The titer of intracellular virus with the +GC strain was maximal 16 hours after infection. Subsequent titration of cell associated virus was very difficult because destructive effects became severe as infection proceeded. However in the —GC strain virus the titer of cells continued to increase after 16 hours.

3. The +GC strain produced giant cells in HeLa cells and L cells as well as in FL cells but L cells disrupted so early after infection that they did not form as large syncytia as those seen with the other two cell strains.

4. When different types of virus were present in a single viral sample, the response of FL cells was influenced by the viral dose.

5. Histochemical changes in infected nuclei were studied.

INTRODUCTION

The formation of intranuclear inclusion bodies in cells infected with herpes simplex virus is recognized as a characteristic change induced by this virus.

Recently, there have been many reports on the growth of herpes simplex virus in various tissue culture cells (Scherer, 1953 ; Scott *et al.*, 1953; Schrer and Syverton, 1954 ; Kaplan, 1957). Studies on the abnormal changes induced by this virus in tissue culture cells revealed various new morphological characteristics which were not recognized in *in vivo* experiments.

Gray *et al.* (1958) reported that infected HeLa cells showed three cytopathic types of response, *e. g.* proliferation, non-proliferation and giant cell formation. More recently, Scott and McLeod (1959) reported further observations on cellular

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responses to infection with herpes simplex virus and distinguished two different virus types, giant cell forming virus and non-giant cell forming virus.

In this laboratory a strain of herpes simplex virus was isolated from the vesicular fluid of a patient with *herpes labialis*, on August 2, 1958 using FL monolayers. It was thereafter serially passaged *in vitro*. To date many experiments, chiefly on the morphological changes of infected cells, have been made with this strain (Nii, 1960 ; Nii *et al.*, 1961). Results are essentially the same as those of Scott *et al.*, although our data differ from theirs on some points.

This report describes the cellular response to herpes simplex virus *in vitro*, showing that it is dependent not only on the strain of virus but also on the type of tissue culture cell.

MATERIALS AND METHODS

1. *Virus*

1) *Miyama strain*

Isolation and biological characters

As already reported, a strain of herpes simplex virus was isolated on FL monolayers from the vesicular fluid of a patient with *herpes labialis*. The patient has subsequently suffered recurrent attacks (Nii, 1960).

This strain can be serially passaged on FL monolayers and causes severe cytopathogenic effects. A neutralization test was used for identification using rabbit antiserum obtained from Dr. Yoshino (NIH, Japan). This strain was designated as the Miyama strain. The culture fluid of infected FL monolayers was used as the viral sample. It was collected when the destructive changes of the monolayer became advanced and was centrifuged at 2,500 rpm for 10 minutes. The supernatant contained about 108 TCID₅₀ per ml.

The biological characteristics of this strain are as follows.

A. When an inoculum derived from the culture fluid was introduced onto the chorioallantoic membrane of 12 day old embryonated eggs, small white pocks were formed by material at an appropriate dilution. Histological examination of the infected membranes showed the formation of intranuclear inclusion bodies. These membranes served as a virus source for subsequent passages.

B. Intracerebral inoculation of a viral sample from infected FL cells into adult mice caused convulsions and death, but if inoculated intraperitoneally the symptoms were slightly different; convulsions occurred first, followed by paralysis of the hind limbs and then death. With the same viral dose lethal effects were less following injection by the latter route. Cerebrum to cerebrum passage was also possible.

C. Ehrlich ascites tumor cells were susceptible to the virus, forming distinct intranuclear inclusion bodies.

D. The host range of the virus was tested with several tissue culture preparations. Infection of HeLa cells and L cells as well as FL cells was possible and all formed intranuclear inclusion bodies.

2) *HF strain*

This strain was obtained from Dr. Yoshino and compared with the Miyama strain.

2. *Cells*

HeLa cells and FL cells were obtained from the National Institute of Health, Japan and Earle's L cells from the Institute for Infectious Diseases, Tokyo. These cell strains have been subcultured in this Institute. 200 ml and 50 ml prescription bottles were used. The L cell strain of mouse fibroblasts was grown in a medium consisting of 95 parts of Hanks' balanced salt solution containing 0.1 per cent Yeast Extract and 0.5 per cent lactalbumin hydrolyzate and 5 parts of bovine serum.

The growth medium of HeLa cells and FL cells was composed of 85 parts of Earle's saline containing 0.5 per cent lactalbumin hydrolyzate and 15 parts of bovine serum. L cell monolayers were prepared in the following way. The maintenance medium of stock cultures was removed from the bottles. L cells were detached from the glass wall of the culture bottles by spraying the wall vigorously with about 5 ml of fresh medium from a bent tipped pipette. Cell suspensions were made by pipetting the liquid back and forth into the pipette. Dispersed cells were diluted to the desired concentration with fresh medium and freshly prepared cell suspensions were introduced into the bottles. After 3 to 4 days, continuous cell sheets were formed. Cell monolayers of FL cells and HeLa cells were prepared from young and vigorously growing cells of continuous stocks. The cell sheet was washed with two changes of Ca- and Mg-free PBS, and the cells were dispersed in a 1 : 20,000 solution of sodium Versenate in Ca- and Mg-free PBS after 5 - 10 minutes' incubation at 37°C. Dispersed cells were sedimented by centrifugation and resuspended to the desired concentration in fresh medium. The resultant diluted suspensions were introduced into bottles or test tubes. Confluent cell sheets were made within 3 days.

For morphological observation of infected cells, monolayers were prepared on 10 × 50 mm coverslips in flat sided test tubes.

3. Infectivity titrations

Titrations of infectivity were carried out in FL cells or L cells by the 50 per cent endpoint method in tube titrations (TCID₅₀). Monolayer cultures were prepared in a series of 15 × 100 mm test tubes containing either 2 × 10⁵ FL cells or L cells. The nutrient media were removed from the test tubes and 0.1 or 0.2 ml of virus sample was added to the cells.

For each dilution three or four test tubes were used. The test tubes were incubated at 37°C to permit adsorption of the virus onto the cells and shaken occasionally to prevent the cells from drying. After 3 hours, 1.5 ml of medium was added to each tube. Cytopathic changes in the monolayers on the test tubes were observed macroscopically and microscopically through the glasswall. Fifty per cent cytopathic doses were calculated by the Reed and Muench method from the number of test tubes showing cytopathic changes within a 6 day observation period.

4. Morphological observations

A. Culture cells were studied under the microscope through the wall of the glass bottle and photographed.

B. Cells grown on coverslips were used to study intranuclear inclusion bodies. They were washed twice with PBS, fixed with Bouin's fluid and stained with Harris' hematoxylin and eosin, or fixed with methanol and stained with Giemsa's solution.

C. The Feulgen reaction was used to study inclusion bodies in cells histochemically.

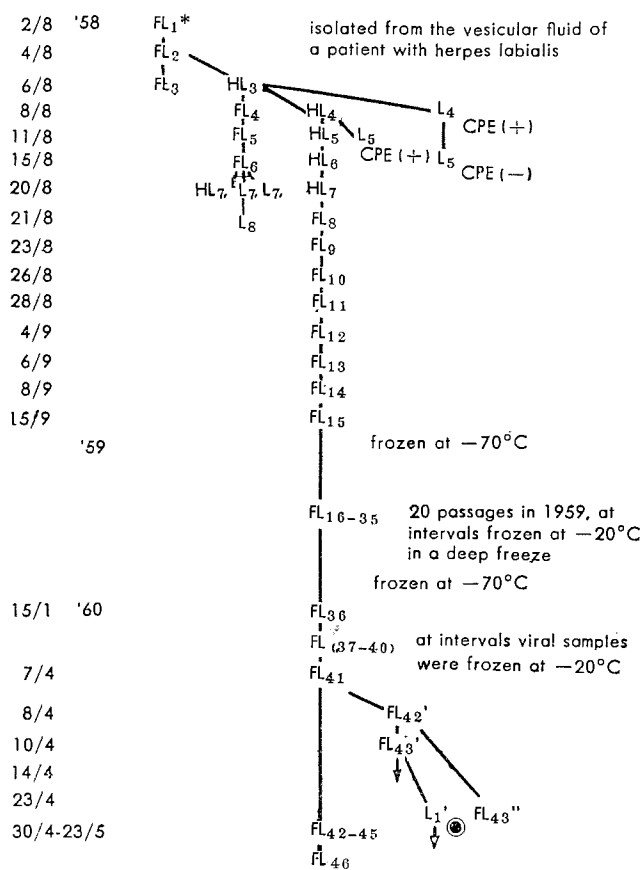
RESULTS

1. Isolation of 2 substrains of the Miyama strain

As described above, the Miyama strain was isolated from a patient onto an FL monolayer. First and second passages on FL monolayers were followed by five passages on HeLa cells. The data from this passage series are shown in Table 1. In each consecutive passage 1 - 2 ml of supernatant fluid of the infected cultures was introduced onto a freshly prepared cell monolayer together with 8 ml of culture medium. Within 20 hours after viral inoculation, most of the cells showed cytopathic effects. The cytopathic changes in infected cells remained the same, *e. g.* rounding for a long period subsequent to isolation of the virus. At the fortieth

passage a new change appeared, namely the appearance of newly formed giant cells with hundreds of nuclei.

Table 1. Isolation and Serial Passages of the Miyama Strain of Herpes Simplex Virus



FL=FL cells HL=HeLa cells L=Earle's L cell

* Number of virus passage

⊙ The culture fluid of the infected FL cells corresponding to the 43rd passage was transferred on an L cell monolayer and then consecutive passages were performed on L cells. The passage number of this series was recorded as L₁', L₂' etc.

In other experiments a series of twenty test tubes were prepared, each tube containing about 2×10^5 FL cells. Viral samples were derived from infected FL cells (No. 47 passage) and tenfold dilutions of the virus were prepared in the medium. Cytopathic changes in a series of test tubes inoculated with viral samples proceeded as shown in Table 2. Of the four test tubes inoculated with 10^{-6} dilutions of virus,

one formed a large syncytial cell sheet covering nearly all the tube : the destructive effect was more severe than in any of the other test tubes which showed cytopathic changes. Although giant cells were formed in the other tubes, they were rather few in number and most of the infected cells appeared only to become rounded. When the culture fluid was removed from the test tube and introduced onto FL monolayers, the cytopathic changes became quite different from those which had been seen during consecutive passages and the 1st +GC virus population was obtained. A comparative study of the morphological changes of infected cells was made using two virus populations ; one was the 1st +GC virus population and the other was derived from virus of the 43rd *in vitro* passage, *e. g.* FL₄₃'. As shown in Figs. 1 and 2, these two populations differed very much from each other in the morphological changes which they produced on FL monolayers.

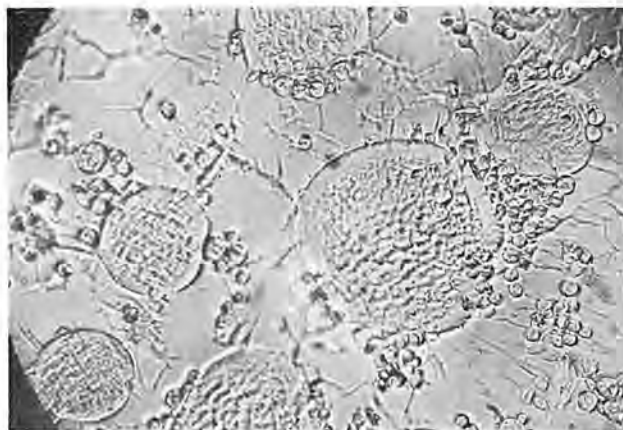


Fig. 1. FL cells infected with +GC virus. 23 hours after infection. ($\times 120$)

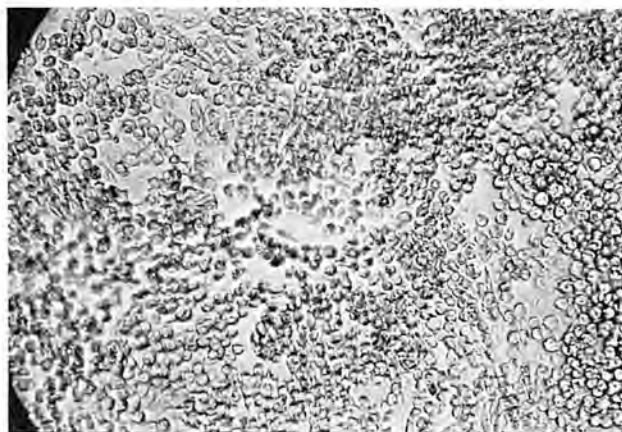
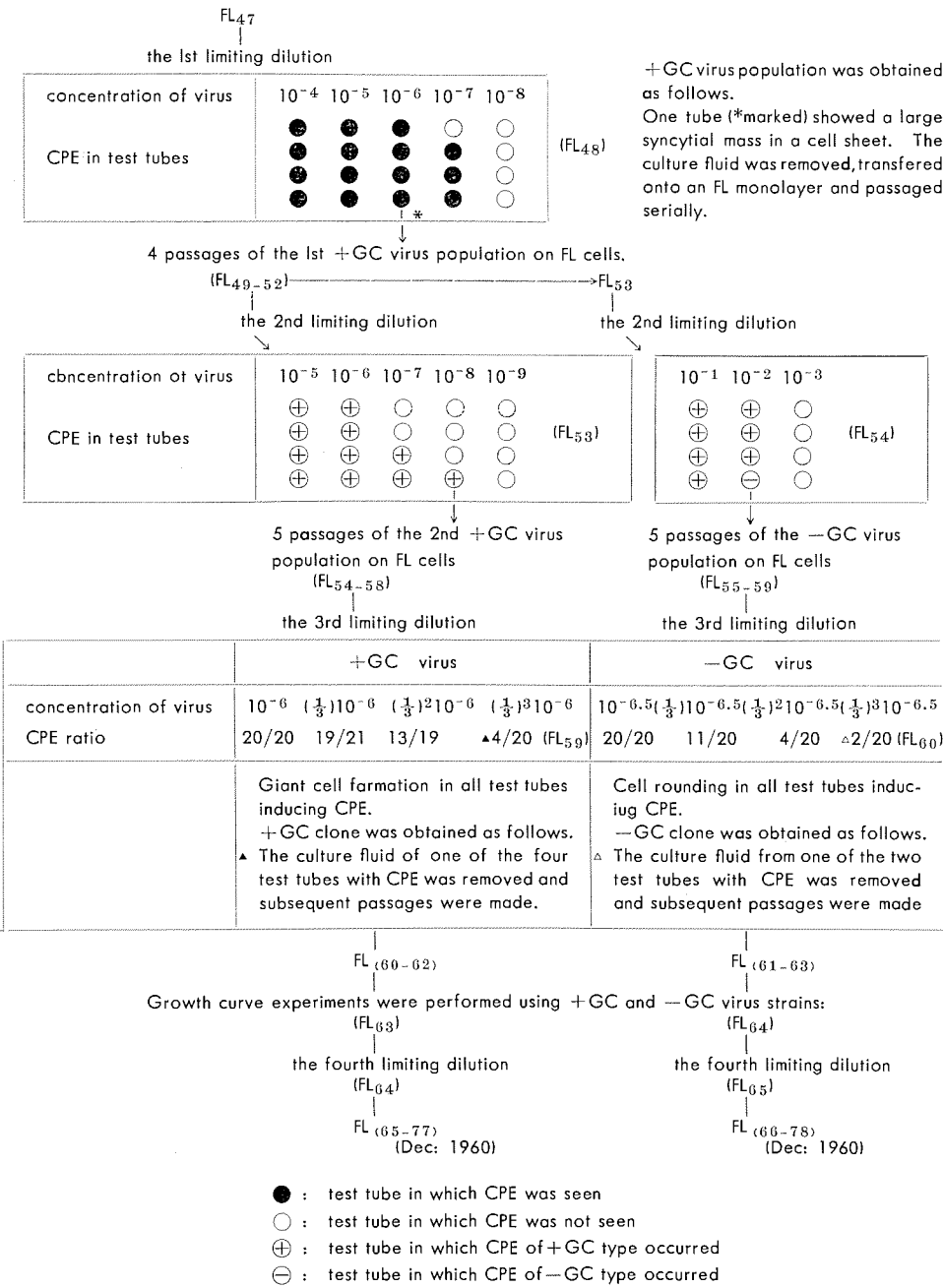


Fig. 2. FL cells infected with -GC virus. 23 hours after infection. ($\times 120$)

Table 2. Isolation of +GC and -GC Virs Strains



The data suggest that cytopathic changes of infected cells depend upon genetic

characters of the virus particles. To ascertain whether giant cell formation in tissue cultures can be used as a genetic marker, a series of isolation procedures was made.

At the end of the 4th passage of the 1st +GC virus population, the growth cycle of herpes simplex virus on FL cells was studied. For titration in this experiment, nine series of test tubes were prepared and for each titration of the nine viral samples one series were used. In Table 2, only two series are listed.

In 95 test tubes cytopathic changes occurred. In 93 there was syncytial formation, while in the remaining 2, cells responded only by rounding. A -GC virus population was obtained from one of the two test tubes.

The data showd -GC virus was yet present in the 1st +GC virus population.

Further large scale purification of each virus population was made. The viral sample of the +GC virus population was diluted serially tenfold up to $1 : 10^6$ and the -GC virus population was diluted up to $1 : 10^{6.5}$. Subsequent dilutions were made by three fold dilutions, as shown in Table 2. 0.1 ml aliquots of each dilution were introduced into about 20 tubes, each containing about 2×10^5 FL cells. Thus four series of tubes were inoculated respectively with four different virus dilutions, and then incubated at 37°C for 3 hours with occasional shaking. After this adsorption period, fresh culture fluid was introduced into the tubes, which were then kept at 37°C for 6 days. Cytopathic changes in the test tubes in each dilution occurred in the ratio shown in Table 3 and the type of change,

Table 3. Virus Particles Necessary for Induction of Cytopathic Changes in Test Tube

Virus dilution	Number of tubes inducing CPE per tubes tested	Multiplicities (Calculated)					
		if ≥ 2		if ≥ 2		if ≥ 3	
		S	$s \times \text{dilution}$	S	$s \times \text{dilution}$	S	$s \times \text{dilution}$
$\times 10^6$	20/20	—	—	—	—	—	—
$\times 3 \cdot 10^6$	19/21	2.35	7.1×10^6	3.95	11.9×10^6	5.39	16.2×10^6
$\times 3^2 \cdot 10^6$	13/19	1.15	10.4×10^6	2.37	21.3×10^6	3.53	31.8×10^6
$\times 3^3 \cdot 10^6$	4/20	0.22	6.0×10^6	0.82	22.1×10^6	1.54	41.6×10^6

S: Multiplicity of infection calculated by Poisson's distribution function from the ratio of infected test tubes in a series
Virus; +GC strain

Virus dilution	Number of tubes inducing CPE per tubes tested	Multiplicities (Calculated)					
		if ≥ 1		if ≥ 2		if ≥ 3	
		S	$s \times \text{dilution}$	S	$s \times \text{dilution}$	S	$s \times \text{dilution}$
$\times 10^{6.5}$	20/20	—	—	—	—	—	—
$\times 3 \cdot 10^{6.5}$	11/20	0.80	$2.4 \times 10^{6.5}$	1.84	$5.5 \times 10^{6.5}$	3.10	$9.3 \times 10^{6.5}$
$\times 3^2 \cdot 10^{6.5}$	4/20	0.22	$2.0 \times 10^{6.5}$	0.82	$7.3 \times 10^{6.5}$	1.53	$13.7 \times 10^{6.5}$
$\times 3^3 \cdot 10^{6.5}$	2/20	0.10	$2.7 \times 10^{6.5}$	0.53	$14.5 \times 10^{6.5}$	1.10	$29.7 \times 10^{6.5}$

Virus; -GC strain

e. g. the +GC type or the -GC type corresponded strictly with the kind of inoculum derived from either the +GC virus population or the -GC virus population.

The ratio of infected tubes in each series indicated the probability that they were infected by the inoculated samples. The relationship between multiplicity of infection and the ratio of infected tubes in a series apparently fitted the Poisson's distribution. As shown in Table 3, a test tube of FL cells suffers cytopathic effects as a result of at least one infectious virus under these experimental conditions. Thus the cytopathic changes occurred in tubes inoculated with the lowest concentration of viral sample may be induced by a single virus particle. The culture fluid of these tubes with low viral inocula was removed and serial passages were made as +GC and -GC clones respectively.

Thereafter these two established virus strains were serially passaged with occasional isolation treatments.

2. *The growth of herpes simplex virus (Miyama strain) on FL monolayers*

As mentioned above, two viral clones were obtained differing in the cytopathic changes produced on FL monolayers. It was of interest to see whether there was any difference in their growth kinetics on FL cells. Three growth curve experiments were performed using viral samples, from the +GC virus population, the +GC virus clone and the -GC virus clone, respectively. The latter two experiments were carried out under comparable conditions to the two strains.

A. *Growth curve of the +GC virus population*

Viral material was derived from the culture fluid of infected FL cells at the fourth passage of the 1st +GC virus population, which corresponded to the 52nd passage *in vitro* after virus isolation.

A 200 ml prescription bottle with a monolayer containing 1.1×10^7 cells, as determined by cell counts from parallel bottles, was washed 3 times with 2 ml of fresh medium and then inoculated with 3 ml of virus sample containing 2.6×10^8 TCID₅₀. After 2 hours incubation at 37°C, the culture was washed 5 times with 5 ml of Hanks' BSS and then 8 ml of fresh medium were added and the bottle again incubated at 37°C. Bottles were removed from the incubator at intervals and the virus, both in the medium and in the cell fractions were titrated as follows. The culture fluid was removed and centrifuged and the supernatant was titrated. To test the infectivity of cell associated virus, cells were washed three times with 3 ml of PBS, suspended in 2 ml of it and disrupted by freezing and thawing; the cell suspension in a test tube was frozen in a dry ice acetone mixture and then the frozen viral material was thawed by plunging the tube into water. The procedure was repeated rapidly six times and the resulting sample was titrated. Its TCID₅₀ was calculated by the method of Reed and Muench.

The results are shown in Fig. 3. The infectivity of the cell fraction indicated an exponential rise between the fourth and eighth hour, while the titer in the

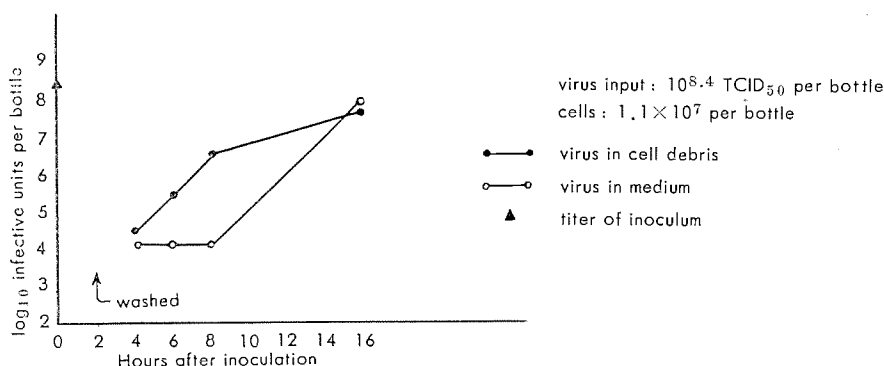


Fig. 3. The Growth of +GC Virus Population in Monolayer Cultures of FL Cells

medium remained unchanged during this period. After 16 hours the titer in the medium was a little higher than after 8 hours and the viral yield per cell was estimated to be about 13 TCID₅₀.

Concurrent morphological changes were also studied. As early as 8 hours after infection, cytoplasmic membranes between neighbouring cells became fused so that the border of each cell was lost and a number of nuclei appeared to be embedded in a large flat cytoplasmic sheet.

B. Growth curve of the +GC strain

A viral sample was obtained from the culture fluid of infected FL cells on passage of strain +GC which corresponded to the 62nd passage after isolation.

Replicate monolayer cultures in 50 ml bottles were prepared. Each bottle contained 2.4×10^6 cells. These were infected with 2 ml of virus suspension of a titer of 2×10^7 TCID₅₀. The cultures were incubated at 37°C for 2 hours with occasional shaking. After the adsorption period, the inoculum was removed. Then monolayers were washed 5 times with 10 ml of Hanks' BSS and 5 ml of fresh medium was introduced onto each monolayer. The bottles were incubated again at 37°C. Two bottles were taken out at intervals from the incubator.

Infectivity of viral samples both in the cellular fractions and in the medium was titrated by the same method used to study the growth of the +GC virus population. When cell suspensions for virus titration in the cellular fraction were prepared, washing of the infected monolayers had to be omitted, because the destructive effects of strain +GC on FL cells were so severe that washing detached the cells from the bottles when cytopathic changes were advanced.

The results are shown in Fig. 4. The infectivity of the cell fraction rose between four and a half and 7 hours and increased exponentially. No lag phase was detectable. The virus titer reached a maximum of 6.3×10^7 TCID₅₀ per bottle at the 16th hour after infection. It decreased between 16 and twenty and a half after infection, perhaps due to cell disruption. The virus titer in the medium

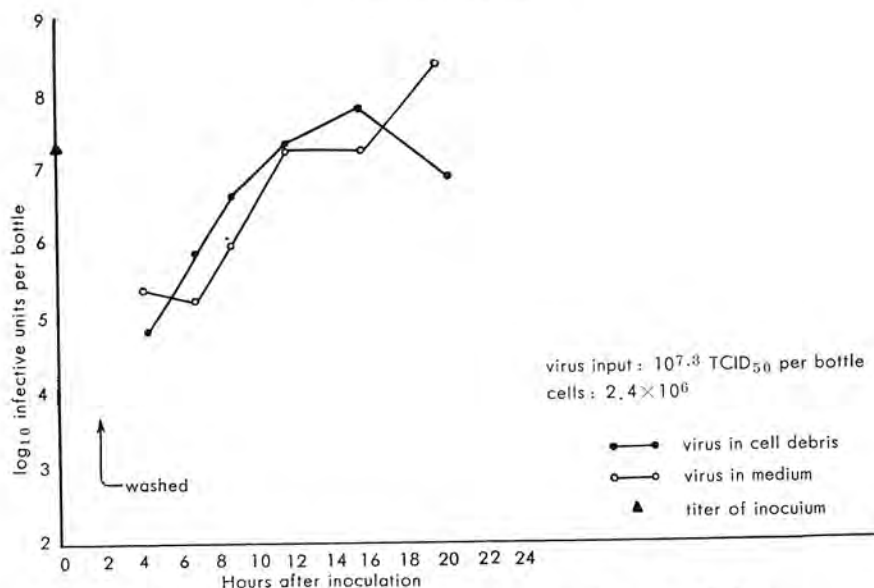


Fig. 4. The Growth of +GC Virus Strain in Monolayer Cultures of FL Cells

rose between 7 and 9 hours after infection and increased exponentially to the 12th hour. It continued to rise after the 16th hour and reached a level of 2.3×10^8 TCID₅₀ per bottle at the 12th hour. The growth curves for the titers in the medium and in the cell fraction crossed between 16 and twenty and a half hours after infection. In this experiment the average viral yield per cell was 100 TCID₅₀.

Concurrent morphological studies were also performed. Cytopathic changes induced by strain +GC appeared to be like those caused by +GC virus population.

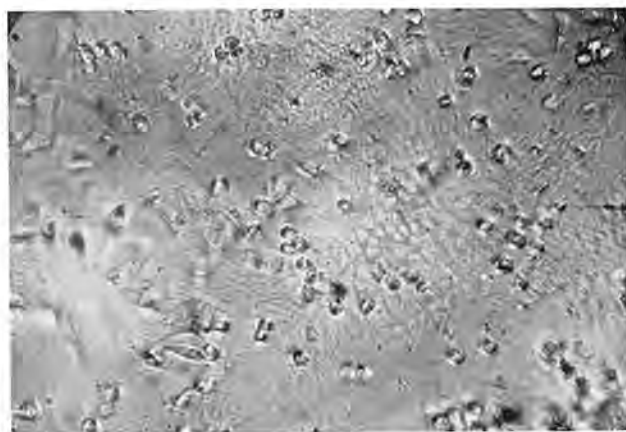


Fig. 5. Monolayer of FL cells infected with the +GC Miyama strain. 9 hours after virus inoculation. A large syncytium of the cell sheet is seen. ($\times 120$)

As early as four and a half hour after infection, there was some breakdown of cytoplasmic membranes between two or three neighbouring cells on the monolayer. The borders of the cells were gradually lost as infection proceeded. At the 9th hour almost all cells in the monolayer had fused into a large syncytium, which spread all over the wall of the bottle and very few cells remained unchanged in the sheet (Fig. 5). Many nuclei appeared faint, due to the difference in their refractive index from that of the syncytial cytoplasm. As infection proceeded, nuclei with rather sharp outlines gathered in one part of the syncytial cell and the rest of the syncytium was devoid of nuclei. The gaps between syncytial sheets became larger and detachment of cell masses began at about 12 hours after infection. At the 20th hour much membranous cell debris had become suspended in the fluid medium and disruption of the syncytium had increased. (Fig. 6)

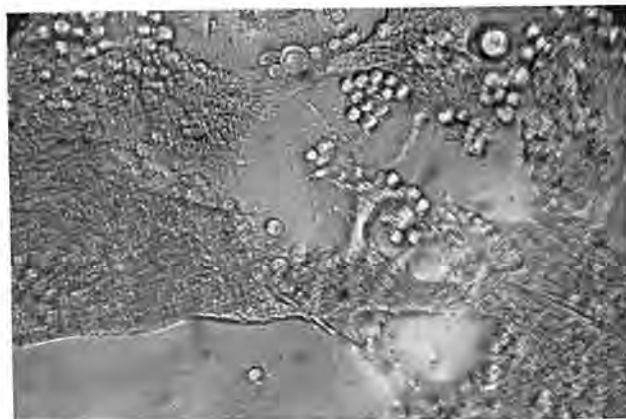


Fig. 6. The destructive effect of the +GC Miyama strain on FL cells. 20 hours after infection. Syncytial cell debris is seen. ($\times 120$)

C. Growth curve of strain -GC

Viral material was derived from the culture fluid of FL cells infected with strain -GC. The virus has been carried through sixtytwo transfers *in vitro* since isolation. The experimental method was the same as that described for experiments with strain +GC.

Replicate monolayer cultures containing 3.6×10^6 cells per bottle were infected with 2 ml of virus having a titer of 2×10^8 TCID₅₀. After two hours adsorption at 37°C the inoculum was removed. The monolayers were then washed 6 times with 15 ml of Hanks' BSS and 5 ml of fresh medium was introduced onto each monolayer. The bottles were incubated again at 37°C and two bottles were taken out at intervals for titration. The results are shown in Fig. 7.

Viral titers in both the cell fraction and the fluid medium decreased between 4 and seven hours forty minutes after infection and then rose. Cell associated

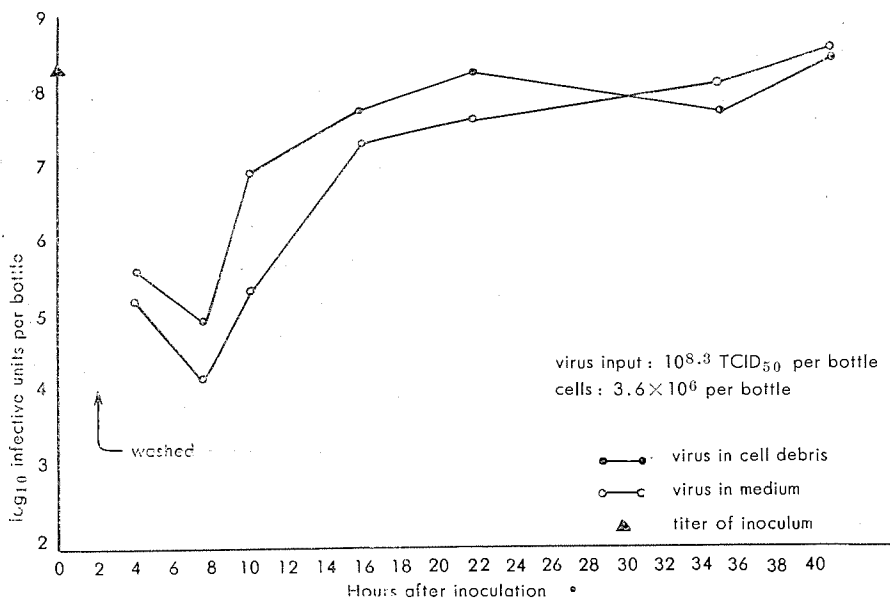


Fig. 7. The Growth of -GC Virus Strain in Monolayer Cultures of FL Cells

virus increased abruptly between seven hours forty minutes and 10 hours after infection and then gradually until the 40th hour, with a slight decrease at the 35th hour. It reached a titer of 4.4×10^8 TCID₅₀.

The virus in the fluid medium increased exponentially between seven hours forty minutes and 16 hours after infection and thereafter rose to a titer of 5×10^8 TCID₅₀ at the 40th hour.

The above data resulted in the crossing of the two growth curves between 22 and 35 hours after infection. The viral yield per bottle at the 40th hour was 9.4×10^8 TCID₅₀ as estimated by the value for cell associated virus titers plus that for the liquid phase. Then average viral yield per cell was found to be about 260 TCID₅₀.

In this experiment, infected cells became round but did not form syncytia and remained attached for 40 hours.

D. Comparative considerations of growth kinetics of the two strains

From the three growth curves described above, various differences between the two strains are apparent.

Although the latent period could not be established exactly, both virus strains seemed to increase in infective titers in cell fractions in nearly the same way up to 16 hours after infection. After about this time the titer of strain +GC decreased due to early cell destruction, while that of strain -GC continued to increase in spite of the higher titer of inoculum per cell used for experiments with this virus. The early disruption of cells by strain +GC was consistently observed in infections

with a high inoculum titer. As shown in Fig. 8, infected cells began to detach like plaques after 12 hours and were much disrupted at about 20 hours.



Fig. 8. The disruption of FL cells infected with +GC and -GC strains. Macroscopic cytopathic changes of monolayer cells cultivated in 200 ml prescription bottles are seen. 20 hours (left) and 12 hours (center) after infection with the +GC strain, 2 days after infection with the -GC strain (right).

The titer of strain +GC increased rapidly in the supernatant fluid and exceeded that in the cell fraction within 20 hours. This was also true 16 hours after infection with +GC virus population containing a little -GC type virus. Early disruption of cells by strain +GC might also be responsible for the early increase in titer in the supernatant.

3. *Neutralization of the two virus strains*

A specific rabbit antiserum against strain HF was used. 0.1 ml of this antiserum neutralized about 100 PFU of strain HF in 0.1 ml of PBS. In this experiment FL cells cultivated in tubes were used. The neutralization indices of the serum with strains +GC and -GC were 2.75 and 2.26 respectively. These values indicated no significant difference between the two viruses with regard to their immunological response to the antiserum.

4. *Difference in susceptibility of L and FL cells to the Miyama strain*

A. *Serial passage of the Miyama strain on L cells (Earle)*

As shown in Tables 1 and 4, consecutive passage of the Miyama strain was performed on L cells. The original viral sample derived from an infected FL culture corresponded to the 43rd passage *in vitro* after isolation from a patient. Subsequent transfer was made as follows; when the cytopathic effects on L cells became advanced, the culture fluid was centrifuged and 0.5 - 1 ml of the supernatant was introduced, together with 8 ml of fresh medium, onto an L cell monolayer containing about 6×10^6 cells in a 200 ml prescription bottle. At each

Table 4. Serial Passage of the Miyama Strain on L Cells

date	FL ₄₃ '	infective titer*
14/4 '60	L ₁ '	8.9×10^5 +
28/4	L ₂ '	1.6×10^6
30/4	L ₃ '	
6/5	L ₄ '	1.6×10^6
7/5	L ₅ '	2.2×10^6
15/5	L ₆ '	1.6×10^4
17/5	L ₇ '	1.6×10^5
24/5	L ₈ '	1.6×10^6
26/5	L ₉ '	1.6×10^5
	L ₁₀ '	1.6×10^6
7/6	L ₁₁ '	2.3×10^6
13/6	L ₁₂ '	1.6×10^7
16/6	L ₁₃ '	
	L ₁₄ '	
22/6	L ₁₅ '	2.3×10^5
25/6	L ₁₆ '	
	L ₁₇ '	
11/7	L ₁₈ '	
18/7	L ₁₉ '	
	L ₂₀ '	
13/8	L ₂₁ '	
13/9	L ₂₂ '	

* Infectivity titrated on FL cells

+ TCID₅₀/ml

Table 5. The Infective Titer of the Culture Fluid of FL Cells, on which Serial Transfers of the Miyama Strain were Made

Exp. 1.*	2.8×10^8 +
Exp. 2.	1.6×10^8
Exp. 3.	1.2×10^8

* Virus from different passages was used in the experiments

+ TCID₅₀/ml

transfer the infectivity of the supernatant was titrated using tubes of FL cells.

The data obtained are shown in Table 4. Serial passage of the Miyama strain on FL cells has already been described. The infective titer of the culture fluids were measured in the same way as those on L cells and was found to be about 10^8 TCID₅₀ per ml. (Table 5) These data showed that L cells yielded 1 - 1.5 log unit per ml less virus than FL cells did.

FL cells were used for the titration because they always showed a higher infectivity titer than L cells.

B. *The morphological response of L cells to the Miyama strain*

During the viral passages described above, infected L cells rounded and became detached from the wall of the culture bottle. The cytopathic changes observed are shown in Fig. 9. Sometimes focal degeneration occurred; many comet-shaped

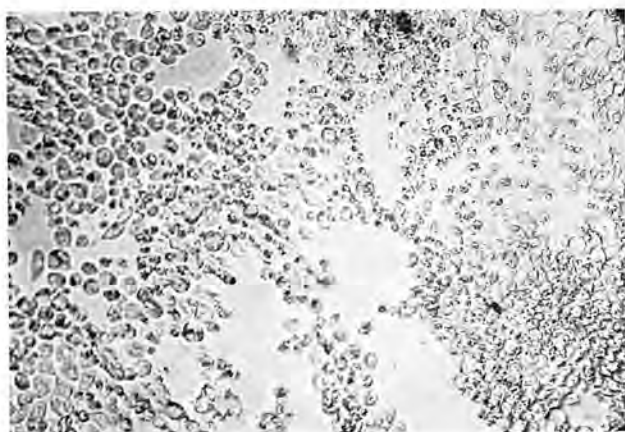


Fig. 9. Cytopathic changes of L cell induced with the Miyama strain of herpes virus. ($\times 120$)

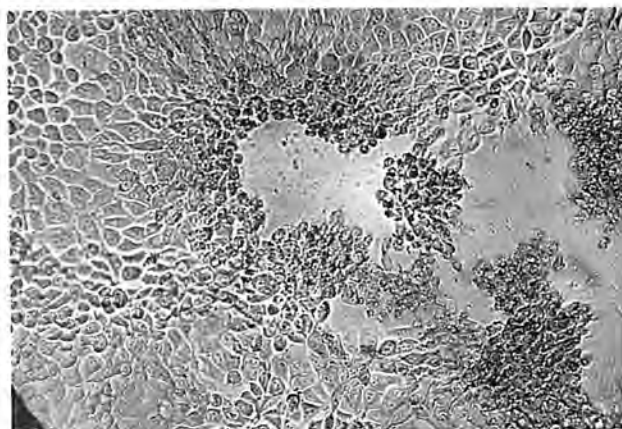


Fig. 10. Focal degeneration on an L cell monolayer infected with the Miyama strain. The cells were cultivated in a 50 ml bottle.

areas with no L cells were observed, as shown in Fig. 10. The direction of the tails of these comets seemed to be influenced by currents in the medium in stationary cultures. Microscopically these areas were surrounded by several layers of rounded cells, adjacent to apparently normal cells (Fig. 11).



Fig. 11. Focal degeneration of an L cell monolayer infected with the Miyama strain of herpes simplex virus. ($\times 120$)

The formation of giant cells was rarely observed during passage of the Miyama strain on L cells but the fact that +GC type virus was present in the viral sample was demonstrated by the following experiments. After 21 consecutive passages on L cells, the culture fluid was transferred again FL cells and serially passaged on the latter. This caused the occasional appearance of giant cells on the infected FL monolayers.

After the 4th, 5th and 6th passage on FL cells of virus transferred from L cells, the supernatant of the culture fluid was appropriately diluted and introduced into a number of test tubes containing FL cells. The resulting cytopathic changes were studied. The results are shown in Table 6. It was found that a little +GC type virus was present in the viral material. When such undiluted viral samples with a titer of about 10^8 TCID₅₀/ml were introduced onto an FL monolayer containing 10^7 cells, giant cells were rarely found and when present, were indistinct. Thus it seems that the formation of giant cells is dependent upon the viral titer of the inoculum.

A further experiment was performed using a +GC virus clone. A high concentration of virus, *e.g.* 10^8 TCID₅₀/ml was inoculated onto an L cell monolayer and caused the formation of many small syncytia in the monolayer. Syncytial cells appeared about 5 hours after infection but L cells were disrupted so early after infection that they did not form as large syncytia as those seen in FL cells.

Thus giant cells are rarely seen during serial passages of the Miyama strain on L cells, firstly because +GC type virus was present in a comparatively low

ratio in the viral sample and secondly because of the early destruction of L cells after infection.

Table 6. Cellular Response to Infection with the Miyama Strain

Virus	Dilution	CPE	CPE containing + GC type response
$\begin{matrix} \text{FL} & \text{HL} & & \text{FL} & & \text{L} & & \text{FL}^+ \\ \text{II} & \text{V} & & \text{XXXVI} & & \text{XXI} & & \text{IV} \end{matrix}$	$(1/3)^2 \ 10^{-6}$	0/22*	0/22
	$(1/3) \ 10^{-6}$	2/25	0/25
	10^{-6}	6/23	2/23
$\begin{matrix} \text{FL} & \text{HL} & & \text{FL} & & \text{L} & & \text{FL} \\ \text{II} & \text{V} & & \text{XXXVI} & & \text{XXI} & & \text{V} \end{matrix}$	10^{-8}	0/11	0/11
	10^{-7}	4/11	0/11
	10^{-6}	10/11	2/11
	10^{-5}	5/5	1/11
$\begin{matrix} \text{FL} & \text{HL} & & \text{FL} & & \text{L} & & \text{FL} \\ \text{II} & \text{V} & & \text{XXXVI} & & \text{XXI} & & \text{VI} \end{matrix}$	10^{-8}	0/6	0/6
	10^{-7}	1/6	0/6
	10^{-6}	6/6	0/6
	10^{-5}	7/7	5/7

These experiments were performed using a series of test tubes of FL cells. Viral samples were prepared by the limiting dilution method.

* $\begin{matrix} \text{FL} & \text{HL} & \text{FL} & & \text{L} & \text{FL} \\ \text{II} & \text{V} & \text{XXXVI} & & \text{XXI} & \text{IV} \end{matrix}$ signify virus serially passaged 2 times on FL cells, 5 times on HeLa cells, 36 times on FL cells, 21 times on L cells and 4 times on FL cells.

* The denominator shows the number of cultures inoculated with the viral sample.

⊙ The numerator shows the number of cultures in which cytopathic effects were seen.

C. Histochemical studies of L and FL cells infected with the Miyama strain

A monolayer containing about 3×10^5 cells on a coverslip was infected with 1 ml of virus of about 10^8 TCID₅₀ titer. After one hour, the inoculum was replaced by fresh medium and the culture was kept at 37°C. To follow the progress of formation of inclusion bodies, infected preparations and controls were fixed with Bouin's solution and stained with Harris' hematoxylin and eosin at various short intervals after infection.

With L cells, the following results were obtained. As early as five hours after infection, a purplish pink homogeneous substance surrounded by coarse strands of chromatin appeared in the nucleus. Pyknosis of some cells was seen within 10 hours after infection. Twelve hours after infection, a variety of changed nuclei were seen, as shown in Fig. 12, some having an inclusion body surrounded by a clear halo, others an incomplete inclusion body filled with a purplish pink homogeneous mass and the rest showing pyknosis. Cells were completely disrupted within 15 hours after infection.

These data suggest that the destructive effect of the Miyama strain on L

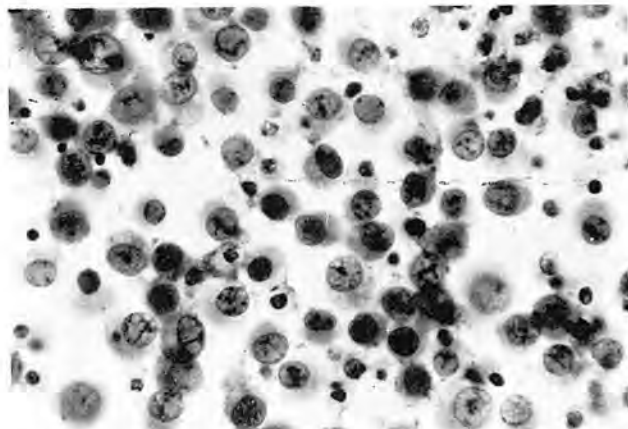


Fig. 12. L cells 12 hours after infection with the Miyama strain of herpes simplex virus. A variety of changes in the cells are shown; intranuclear inclusion bodies and nuclei filled with an eosinophilic mass stained bluish pink. Fixed with Bouin's solution and stained with hematoxylin and eosin. ($\times 650$)

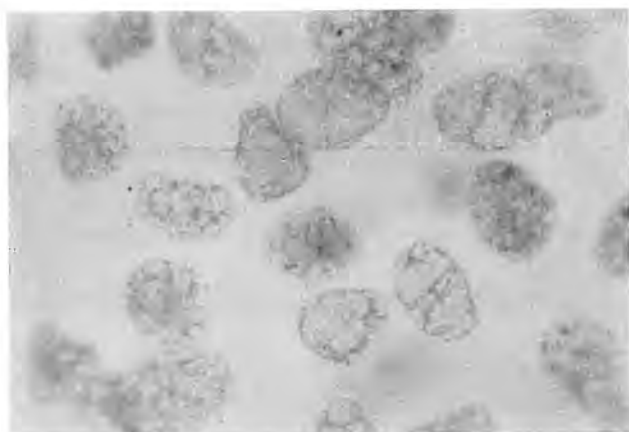


Fig. 13. L cells 12 hours after infection with a high titer of the Miyama strain of herpes virus. After fixation with methanol, the Feulgen reaction was performed. ($\times 1600$)

cells was so severe that complete development of inclusion bodies might be inhibited. Cells showed pyknosis as soon as they reached the stage with bluish pink homogeneous bodies in the nuclei of cells in stained preparations.

FL cells infected with the virus showed a more synchronous development of inclusions and the developmental period lasted longer. Twelve hours after infection all cells contained homogeneous bodies or typical eosinophilic inclusion bodies surrounded by clear halos. Disruption of cells began about 20 hours after infection with strain +GC as described above, but was delayed with strain -GC.

Infected cells were also stained by the Feulgen method after fixation in metha-

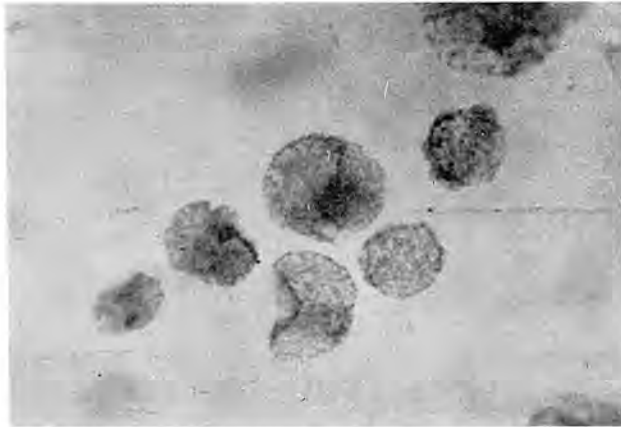


Fig. 14. FL cells 12 hours after infection with a high titer of the Miyama strain of herpes virus. After fixation with methanol, the Feulgen reaction was performed. ($\times 1600$)

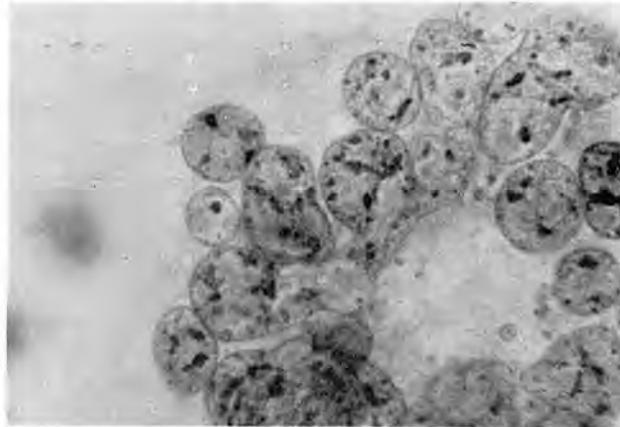


Fig. 15. Giant cells from monolayer cultures of L cells infected with the HF strain of herpes virus. A number of nuclei are connected together by fine bridges and globules are scattered in the cytoplasm of the giant cells. ($\times 1600$)

nol. As shown in Figs. 13 and 14, the nuclei of L cells 12 hours after infection were positive and had a finely homogeneous appearance, while FL cells at the same period after infection had a rather coarse appearance.

5. *Other studies on the cellular response to infection with herpes simplex virus*

The response of HeLa cells to two types of the Miyama strain was studied. Both types of virus were introduced onto HeLa cells and the latter showed cytopathic changes corresponding to the type of the virus, as in FL cells.

For comparison with the Miyama strain, strain HF obtained from Dr. Yoshino was used. The cytopathic changes in FL and L cells were studied. Many giant

cells with up to twenty nuclei were found in L cell monolayers, while FL cells responded by rounding and occasionally formed small giant cells. In L cells infected with strain HF some giant cells had a peculiar shape, as shown in Fig. 15; a number of nuclei became clustered in the syncytium and fused with each other with nuclear bridges. In such nuclei inclusion bodies or bluish pink masses were usually, but not always, found. Eosinophilic globules which were thought to be formed by amitotic division, were scattered about in the cytoplasm.

DISCUSSION

Many varieties of host cells and virus strains have been used to study the growth of herpes simplex virus. The most characteristic cytopathic change induced by this virus is the formation of intranuclear inclusion bodies in infected cells. Giant cell formation in monolayer cells infected with the agent has been reported by several workers (Newton, 1958; Ross and Orlans, 1958). Giant cell formation as a response to virus infection has been reported with many other viruses, *e. g.* measles, herpes B, pseudorabies, HVJ and some pox viruses (Enders, 1954; Black and Melnick, 1955; Okada, 1958; Nii, 1959). Studies on the formation of giant cells as a genetical character of virus particles was reported for measles and pseudorabies (Seligman and Rapp, 1959; Tokumaru, 1957). Scott *et al.* (1959) recently described two types of herpes simplex virus, strains GC and P, the one producing giant cells and the other not.

We independently obtained similar results during a study of the cellular response to a newly isolated herpes simplex virus *in vitro*. Since isolation of the Miyama strain of herpes simplex virus onto an FL cell monolayer, viral passages have been repeated *in vitro*. In earlier generations of passage infected cells only became rounded, but later giant cell formation occurred. These successive passages *in vitro* ultimately resulted in the isolation of two sub-strains, *e. g.* strains +GC and -GC, which were distinguishable by the effects they induced. Therefore type +GC virus must have increased in number in the parent virus population during serial passage, although the origin of the +GC virus is unknown. Microscopic plaques formed on a monolayer inoculated with a lower concentration of the Miyama strain were studied and the ratio of the constituent virus particles, such as types +GC, -GC in the parent viral population were studied. Farnham reported that a strict proportionality existed between the plaque number on the third day and the virus input in the absence of an agar overlay or herpes antiserum. She also observed two distinct features of microscopic plaques; the one was clusters of rounded, highly refractive cells and the other was multinucleated giant cells. In our system, plaques counted on the 2nd day was approximately proportional to the virus concentration but on the 3rd day counting was difficult, as the primary plaques became diffuse and in addition small satellite plaques had appeared. +GC type virus forms a plaque consisting of a single giant cell at an early stage

of infection, while the plaque by —GC virus is chiefly composed of rounded cells. In a series of experiments using the Miyama strain, it was noted that in early generations of passage after isolation, a number of microscopic plaques of round cell types were seen on FL cells but the +GC type failed to show an effect, suggesting that there was little if any +GC virus.

The question therefore arises as to what regulates the increase in +GC type virus in the parent virus population. Growth experiments showed that +GC type virus caused early destruction of FL cells and attained a little higher titer in the culture fluid than did the —GC type virus. However, it is not certain whether the former factor is advantageous in causing an increase in the number of +GC type over the parent —GC viral population during serial passages *in vitro*.

In our system the infectivity in the culture fluids of FL cells infected with both types of virus rose sharply and was more than 1 TCID₅₀ per cell between 12 and 16 hours after infection. It then reached a maximum titer of 100 TCID₅₀ or more per cell. The titer in the culture fluid of FL cells infected with —GC virus did not decrease for 40 hours, although initially there was a high virus input. This result differs from that of Gray *et al.* (1958).

The virus titers on FL monolayers and chorioallantoic membranes of chick embryos was compared. The Miyama strain which had been passaged 63 times *in vitro* after isolation without an egg passage was almost as infectious to FL cells as to the chorioallantois. ($\log_{10}$ 50% cytopathic dose on FL cells: pock units on the chorioallantoic membrane = 1.8)

A comparative study of the susceptibility of FL and L cells with the Miyama strain was described. There was a lower yield of virus in L cells. The same phenomenon has been reported by McClain and Hackett with vesicular stomatitis viruses. Barski (1960) also found that Earle's L cells were less susceptible to ectromelia virus than human epithelial cells.

When +GC viruses derived from FL cell-passage, were introduced onto an L cell monolayer, the cells became disrupted, producing many small syncytial cells. However, large syncytia like those seen in FL cells did not appear. Infected L cells became disrupted much earlier than FL cells when inoculated with the same amount of the Miyama strain. The response of HeLa cells to +GC and —GC strains resembled that of FL cells. These data showed that the cellular response to infection with herpes simplex virus were influenced by the type of host cell. Another factor was found which played a role in the appearance of giant cells. When a high viral dose containing a low ratio of +GC virus was introduced onto FL monolayers the cytopathic change was a —GC type response. With a low viral dose, however, there was a +GC type response. The HF strain was also tested on FL and L monolayers and the cytopathogenic change induced was found to differ from that of the Miyama strain.

Crandell (1959) studied the conditions which influence the type of cell res-

ponse to a strain of herpes simplex virus. Although he suggested that there might be a mixture of cytopathogenic types of virus he made no attempt to isolate the various types. In this report we have distinguished two types of viruses from their cellular responses. There may also be other types of the Miyama strain.

The formation of intranuclear inclusion bodies in infected cells was also studied. Well developed inclusion bodies were seen in FL cells, while L cells disrupted as soon as their nuclei contained inclusion bodies.

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