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Host-Virus Interactions during Infection with Herpes Simplex Virus

I. Growth Characteristics of the Miyama Strain of Herpes Simplex Virus in L Cells

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SUMMARY

Analytical studies were made on the low susceptibility of Earle's L cells to the -GCr Miyama strain of herpes simplex virus. In comparative titrations ($TCID_{50}$) using FL and Earle's L cells, the latter cell line always showed a lower infective titer than the former. However, approximately the same number of inclusions were detected in the two cell lines between 8 and 9 hours after virus inoculation. The number of infective centers was counted in both cell lines after inoculation with the same dose of virus and it was found that FL cells produced at least one log unit more infective centers than did Earle's L cells. Therefore apparently only some infected L cells can release extracellular infective virus and other cells may show "abortive infection", resulting in inclusions in the nuclei.

In L cell monolayers inoculated with a low virus multiplicity, the titer of extracellular virus rose one or two days after infection and thereafter decreased. This was partly due to the low viral yield of L cells and partly to abortive infection in some cells with inclusions. Other explanations for this phenomenon, such as a lysogenic effect or autointerference seem unlikely.

INTRODUCTION

Studies on the growth of herpes simplex virus *in vitro* using L cells were reported by Scherer (1953), Powell (1959) and Nii *et al.* (1961). Our previous experiments showed that L cells were less susceptible than FL cells to the Miyama strain of herpes simplex virus. Our previous data are summarized as follows: 1) Using human vesicular fluid isolation of the Miyama strain virus of herpes simplex onto L cells was unsuccessfull but onto FL cells it was successfull. 2) Attempts at serial transfer of virus on L cells were usually unsuccessfull. In one case only, 22 serial transfers over the half year experimental period were possible. In these passages lower viral yields were obtained from the culture fluids than from those of FL cells.

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3) In simultaneous titrations using the same dose of the virus, L cells always gave a lower titer than FL cells.

There are various possibilities, why L cells show a lower susceptibility to the virus than FL cells. The first is that there may be a difference between the process of virus adsorption onto the two types of cells. The second possibility is that only a few per cent of L cells may be able to initiate further steps of virus reproduction after virus adsorption for some physiological reason. The third possibility is that some steps in the process of virus reproduction may be inhibited or arrested in the cells for some reasons. The fourth possibility is that virus growth and the appearance of cytopathic changes in L cell monolayers may be extremely slow because of the low viral yield per cell.

In our earlier paper we reported that one infectious virus particle may be sufficient to infect an FL cell and to cause the formation of an intranuclear inclusion in it (Nii *et al.*, 1962).

This paper describes some analytical studies at the cellular level on the lower susceptibility of L than FL cells using the formation of intranuclear inclusion bodies as an index of susceptibility.

MATERIALS AND METHODS

1. *Virus*

-GCr Miyama strain was used (Nii *et al.*, 1961). The culture fluid of infected FL monolayers served as the viral sample. It was collected when cytopathic changes in the monolayer became advanced and was centrifuged at 2,500 rpm for 10 minutes. The supernatant contained about 10^8 $TCID_{50}$/ml.

2. *Cells*

Both FL and L cells were prepared on 10×40mm coverslips in square tubes. Two or three days after planting, monolayers were obtained, each containing $5 \times 10^5 \sim 1 \times 10^6$ cells. These were used for experiments.

3. *Culture media*

Earle's L cells of mouse fibroblasts were 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 FL cells was composed of 90 parts of Earle's saline containing 0.5 per cent lactalbumin hydrolyzate and 10 parts of bovine serum.

4. *Cell counting*

The number of cells on coverslips was counted as described in the earlier report (Nii *et al.*, 1962). Cells in the bottles were counted in the following way. After discarding the culture fluid from the bottles, 5 ml of 0.1 M citric acid was introduced into each bottle, and the bottles were incubated at 37°C for 1 hour. Then the cells were detached from the glass wall by spraying the wall vigorously with the liquid. After the liquid had been pippetted back and forth, the cell nuclei could be sedimented by centrifugation at $600 \times g$ for 10 minutes. They were resuspended in 2 ml of 0.1 M citric acid containing 0.01 per cent gentian violet and 0.25 per cent methylcellulose. Cells were counted in a haemocytomer.

5. *Virus infectivity titration* ($TCID_{50}$)

Essentially the same method as described previously was adopted (Nii *et al.*, 1961). At each

dilution four test tubes were used. The test tubes were incubated at 37°C to permit adsorption of the virus onto the cells and were agitated intermittently. After 1 hour, 1.5 ml of maintenance medium was added to each tube. Cytopathic changes in the monolayers on the test tubes were observed macroscopically and microscopically through the glass wall. Fifty per cent cytopathic doses were calculated by the Reed and Muench method from the number of test tubes showing cytopathic changes during a week's observation period.

6. *Counting of inclusion bearing cells in the sheet*

The method reported previously was adopted (Nii *et al.*, 1962). The method of studying inclusion body development in L cells was also as described previously.

7. *Number of infective centers*

Monolayers were inoculated with 2 ml of virus. After an hour's adsorption period, the inoculum was discarded and the monolayers were washed 7 times with 2 ml of Hanks' BSS. Then cell suspensions were made. FL cell suspensions were prepared by EDTA treatment, while those of L cells were made by pippetting in YLH solution (Hanks' BSS containing 0.1 per cent Yeast Extract and 0.5 per cent lactalbumin hydrolyzate). The FL cells were finally suspended in 5 ml of LE and L cells were suspended in the same volume of YLH. The number of cells was counted in a haemocytometer. These cell suspensions were diluted serially tenfold; the sample of L cells was diluted with YLH and that of FL cells with LE. The diluted suspensions were inoculated onto the FL cell monolayers used as indicators. Aliquots of 0.1 ml of each diluted suspension were inoculated into four test tubes. Cytopathic changes in the monolayers were observed over a period of a week. Fifty per cent cytopathic doses were calculated by Reed and Muench's method.

RESULTS

1. *Comparative titrations ($TCID_{50}$) on FL and Earle's L cells*

Two series of test tubes each containing a monolayer of FL or L cells were prepared. The virus samples were made by the tenfold dilution method and the same virus materials were inoculated onto each of the two monolayer series. For each a week's observation period was adopted.

Positive or negative signs of infection in tubes were ascertained as follows. Tubes revealing definite cytopathic changes macroscopically as well as microscopically were regarded as showing positive signs of infection. Therefore, tubes in which focal lesions on the monolayers were found microscopically, but in which

Table 1. Titration of the Miyama Strain of Herpes Simplex Virus on FL and L Cells

	FL cells	L cells	$\log_{10}$ difference
Parent virus population	5.5	4.8	0.7
	7.5	5.5	2.0
−GCr substrain	6.5	5.3	1.2
	8.3	3.5	4.8
+GC substrain	8.3	3.5	4.8
	7.5	2.5	5.0
	6.5	2.5	4.0
	7.3	2.5	4.8

$\log_{10}$ infective units ($TCID_{50}$) per ml

no signs of infection such as focal degeneration or detachment of cells could be seen with the naked eye, were regarded as showing negative infection.

By this method, FL cells always gave a higher infective titer than L cells. This in shown in Table 1.

2. *Appearance of intranuclear inclusions in Earle's L cells*

It is supposed that the appearance of inclusion bodies induced by herpes simplex virus is more or less dependant on host cells or virus strains and such morphological mainfestations may be closely correlated with virus multiplication in the cells. From earlier studies using FL cells, we reported that the number of inclusion bearing cells detected between 8 and 9 hours after virus inoculation approximately fitted a Poisson's distribution curve, assuming a one hit event. This section describes the development of inclusion formation on L cells and a comparison of their development with that on FL cells. The same experimental

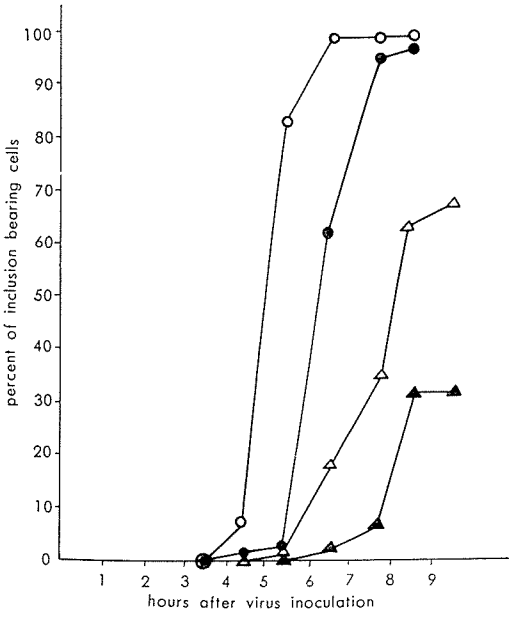

Fig. 1 Inclusion Development (—GCr Miyama Strain : L Cells)

○——○ inoculated with a virus dilution of $1 : 3^0$
●——● inoculated with a virus dilution of $1 : 3^1$
△——△ inoculated with a virus dilution of $1 : 3^2$
▲——▲ inoculated with a virus dilution of $1 : 3^3$
adsorption period : one hour

procedure was used for both L and FL cells as described in Materials and Methods. The results are shown in Fig. 1.

The curves for development of inclusions in cells inoculated with higher diln-

tions of virus indicate a shoulder from the 8th hour after virus inoculation. This suggests that the first cycle of appearance of inclusions and the mode of their apprarance are approximately the same in FL and L cells.

However, some morphological differences in the inclusion bodies in FL and L cells were noticed. Generally speaking, formation of eosinophilic masses is not so great in L cells as in FL cells. It is a well known fact that halo formation of inclusion bodies is dependent on fixatives. Halo formation of inclusions seems to be influenced more or less by the adsorbed virus multiplicity on L cells but this was not apparent in FL cells, the nuclei of which always formed a dense eosionophilic substance after infection.

The cells, which were counted as inclusion bearing cells in the infected L cell monolayers, showed one of the following morphological changes:

1) definite eosinophilic masses, which occupied part of the nucleus, with or without a halo.

2) nuclei filled with a purplish pink mass, without formation of a halo.

3) definite condensation of chromatin in the nuclei, which often contained small eosinophilic masses.

4) eosinophilic masses, which occupied the whole of the nucleus, with clear halos.

Similar experiments also showed only a slight increment in the number of inclusion bearing cells between 8 and 10 hours after infection in cultures inoculated with higher dilutions of virus. It would be interesting to know the time when recycling occurs, but destruction of infected L cells and their detachment from the glass wall occurred so rapidly (*i. e.*, 12 hours after infection) that it was difficult to follow subsequent cellular changes quantitatively.

3. *Comparison of the number of inclusion bearing cells in infected FL and L cell monolayers*

This article describes comparative studies on the number of cells in the two types of monolayers affected by primary input virus.

The percent of inclusion bearing cells detected between 8 and 9 hours after infection in the two types of cell monolayers was calculated by counting more than one thousand cells and the adsorbed virus multiplicity was calculated from Poisson's formula assuming a one hit event. The number of cells on the coverslips of parallel cultures was counted and thus the number of inclusion forming units per coverslip was obtained from the product of the adsorbed multiplicity and the number of cells.

The supernatant fluid of infected FL cells was used as the viral source. This undiluted original viral sample contained approximately 10^8 $TCID_{50}$ per ml and therefore in monolayers inoculated with low virus dilutions almost all the cells usually contained inclusions.

Using diluted samples, however, both affected and unaffected cells were found in the monolayers and it was possible to calculate the adsorbed viral multiplicity. In this way the adsorbed viral titer of the undiluted original viral samples was

calculated by multiplying the titers by the dilution factor.

Table 2. Number of Inclusion Forming Units per Coverslip in FL and L Cell Monolayers

	FL cells	L cells
Exp. 1	6.5×10^6	6.2×10^6
Exp. 2	2.2×10^5	2.4×10^5
Exp. 3	8.5×10^6	8.4×10^6

As shown in Table 2, about the same numbers of I. F. U. per coverslip were found in the two cell lines. This means that virus of the —GCr Miyama strain was adsorbed to the same extent by FL and L cells to induce subsequent morphological changes in them.

4. *Comparison of the number of infective centers in FL and L cells inoculated with a low multiplicity of virus*

This section describes studies on whether all, or only some, of the infected L cells which eventually show morphological changes in their nuclei release infective virus particles. In earlier studies on FL cells, it was found that the number of inclusion forming units per coverslip was equal to the number of microplaque forming units, and therefore an infective virus particle induced intranuclear inclusions in an FL cell and this initiated the subsequent cycle of virus growth and released extracellular viruses forming a microplaque in the monolayer.

Both FL and L cell monolayers were prepared in 50 ml bottles and a monolayer sheet having $3-5\times10^6$ cells was used in each experiment. It was considered that about the same number of cells were present in each bottle. Virus of a low titer was used so that multiple infection of the cells in the sheet could be avoided. Monolayers were inoculated with 2 ml of virus. After an hour's adsorption period, the inoculum was discarded and then the monolayers were washed. FL and L cell suspensions were prepared as described in Materials and Methods and were diluted serially tenfold. Diluted samples of both cell lines were inoculated on the FL cell monolayers used as indicators.

Table 3. Number of Infective Centers Formed by FL and L Cells

	titer of input virus (per 2 ml)	number of infective centers in FL cells	number of infective centers in L cells
Exp. 1	$10^{6.6}$	$10^{6.1}$	$10^{4.9}$
Exp. 2	$10^{7.5}$	$10^{6.9}$	$10^{5.4}$

As shown in Table 3, FL cells produced one log unit, or more, more infective centers than L cells, while it has already been shown that the numbers of inclusion

bearing cells were almost equal in the two cell lines when the cells were exposed to the same virus input. Therefore it is deduced that only a low proportion of infected L cells can release extracellular infective virus and other cells show "abortive infection" resulting in morphological changes in the nuclei.

5. *Growth experiments with —GCr virus with a high virus input on L cell monolayers*

In L cell monolayers infected with a low virus input, cytopathic changes are very delayed or arrested. This is firstly due to abortive infection, as described above, and another factor may be related to the viral yield from L cells. The experiments described below were to study the average viral yield per cell.

L cell monolayers were prepared. Each monolayer contained approximately 4.4×10^6 cells per bottle. Two ml of —GCr virus of 10^8 $TCID_{50}$ were inoculated on each monolayer and an hour's adsorption period at 37°C was chosen. Monolayers were washed five times with 5 ml of Hanks' BSS and then 5 ml of medium was introduced. At intervals after infection a bottle was taken out from the incubator to measure the infective titer of the liquid phase and cell-associated virus.

The results are shown in Fig. 2. The titer of cell associated virus rose rapidly between 7 and 9 hours after infection and reached a maximum at the 15th hour,

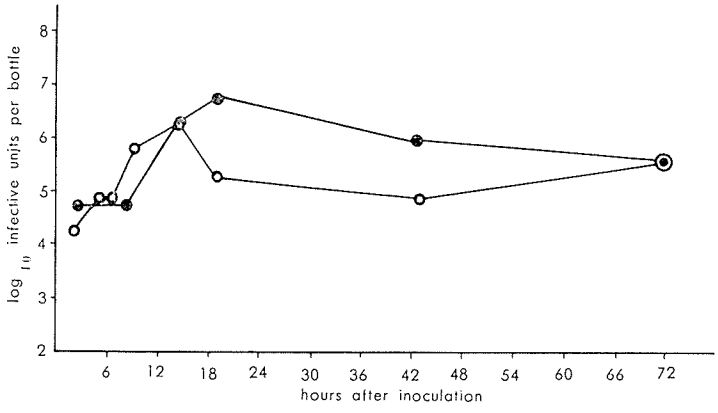

Fig. 2 Growth of the —GCr Miyama Strain on L Cell Monolayers

○——○ cell associated virus

●——● virus in culture fluid

virus input : $10^8 TCID_{50}$ per 2 ml

cells : 4.4×10^6

subsequently decreasing until the 19th hour. Thereafter it remained at approximately the same level for the next 2 days. The titer of the supernatant fluid showed a definite rise from the 9th hour after infection. This was confirmed in a repeat experiment. (Fig. 3). The titer of the culture fluid at the 19th hour was 5×10^6 $TCID_{50}$ per bottle and therefore a viral yield of 1 or more $TCID_{50}$ per cell was

obtained. The yield, however, was very low compared with that from FL cells, which produced 200 or more $TCID_{50}$ per cell.

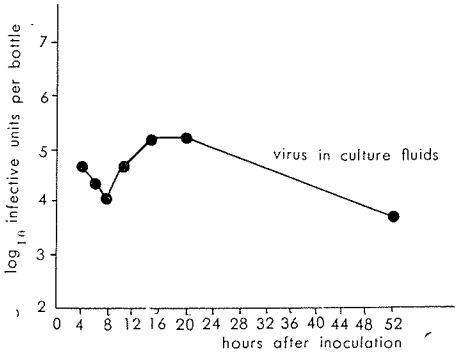

Fig. 3 Growth of The —GCr Miyama Strain on L Cell Monolayers

6. *Response of L cells to infection with —GCr Miyama strain of herpes simplex virus*

a) *Cellular response to high virus input*

Experiments described above show that with —GCr Miyama strain, L cells given a low viral yield per cell and that some infected cells can release infective virus at the infection of low virus multiplicity. Another biological character of L cells, which may have a close relation with these phenomana is described below.

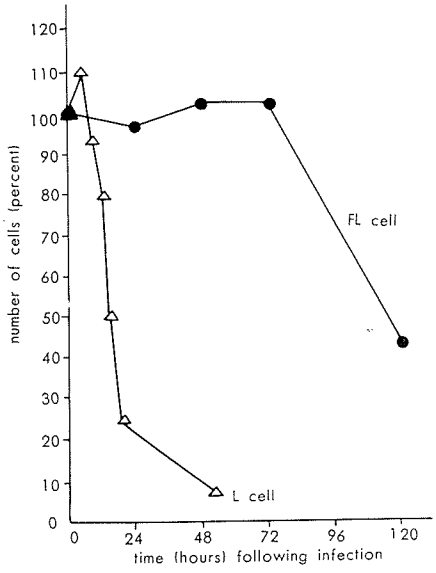

Fig. 4 Cell Response to a High Virus Input of the —GCr Miyama Strain of Herpes Simplex Virus

Two ml aliquots of viral sample, of approximately 2×10^8 $TCID_{50}$, were introduced onto a series of monolayers each containing 3.4×10^6 cells. After an hour's adsorption period at 37°C, the inoculum was discarded and the monolayer washed 5 times, with 2 ml aliquots of Hanks' BSS. Then the medium was introduced. At intervals, a bottle was taken out of the incubator and after discarding the medium, 5 ml of 0.1 M citric acid was introduced. Cell counting was performed as described in Materials and Methods. In this way the number of cells remaining attached to the glass wall after infection was followed.

As shown in Fig. 4, half the monolayer cells were lost by the 15th hour after infection. This is in sharp contrast to results of a similar experiment using FL cells, in which there was little change in the number of cells during the first 3 days after infection.

In stained preparations of L cells inoculated with a high viral dose, pyknotic nuclei were found from the 10th hour after infection and thereafter they gradually increased in number.

Early disruption of the L cells may prevent further virus maturation in them and this may be a cause of the low virus yield and abortive infection with L cells.

b) *Dose response growth curves of —GCr virus on L cell monolayers and concurrent cellular responses*

Three viral concentrations, containing 10^8, 10^6 and 10^4 $TCID_{50}$ per 2 ml, respectively, were prepared. Two ml aliquots of these samples were introduced into three series of bottles, each containing 3.0×10^6 cells. The bottles were then incubated at 37°C for 2 hours.

After the adsorption period, the inocula were discarded, the monolayers were washed well and then medium was introduced. At appropriate intervals a bottle

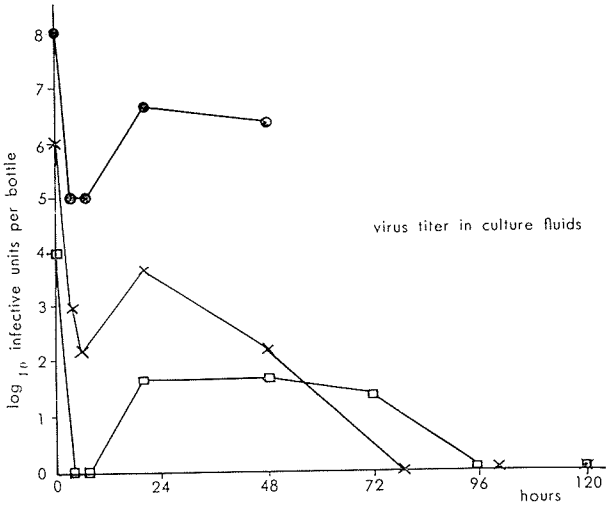

Fig. 5 The Growth of —GCr Miyama Strain on L cell Monolayers

from each series was taken out of the incubator and the culture fluid was removed to titrate on FL monolayers. At the same time the number of cells remaining on the bottle wall was counted.

The results are shown in Fig. 5. After initial low titers between 4 and 6 hours after infection the curves all rose to maxima at the 20th hour after infection. The maximum titer of each series, however, was lower than that of initial input virus added. In the series of cultures infected with the highest viral dose, the experiment was stopped at the 48th hour after infection. The two curves for low viral doses decreased after the maximum and four days after infection no virus could be detected. The medium was changed on the 5th day after infection and thereafter daily. In the cultures inoculated with the highest viral dose, total cell degeneration occurred, while in the other two series of cultures there was an increase in the number of cells and the series of cultures inoculated with the lowest virus input always contained the most cells. In the cultures inoculated with two ml of 10^6 $TCID_{50}$ of virus, focal lesions were sometimes seen by microscope, but finally they could not be distinguished due to cell proliferation. Further change of the medium did not induce extensive cytopathic changes.

c) *Cellular responses to various doses of virus*

The relation of viral growth to cellular response is presented above. Further experiments on the latter were performed using various doses of virus per 2 ml. Six different viral samples were made by the three fold dilution method. Two ml aliquots of each viral sample were inoculated into a series of bottles. After a 2 hour's adsorption period, the inoculum was replaced by medium. Subsequent cellular responses in each series of cultures were followed at appropriate intervals after inoculation. The results are shown in Fig. 6. In the cultures inoculated with an input virus multiplicity of more than 1, total degeneration of cells occurred. As input multiplicities decreased below 1, cytopathic changes decreased and the cells proliferated. Subcultures were made of these proliferating cells. When these were exposed to a high titer of virus of approximately 10^8 $TCID_{50}$, they were destroyed completely. However, no phenomenon analogous to autointerference was demonstrated.

d) *Effect of cellular conditions on viral growth*

In a system of L cells and the Miyama strain of herpes simplex virus, viral growth seemed to be more or less influenced by the physiological conditions of the cells. The latter may be related to the days after cell seeding and therefore three different cellular conditions were chosen to study the effect as follows. One series consisted of two day old cultures and the second series of four days old cultures. These were both infected by the routine method. The third series of cultures were inoculated with virus at the time of cell planting. In the former two series, each culture contained $3-4\times10^6$ cells and was inoculated with 2 ml of virus of 10^6 $TCID_{50}$. In the third series of cultures, one and a half ml of virus of 2.5×10^6

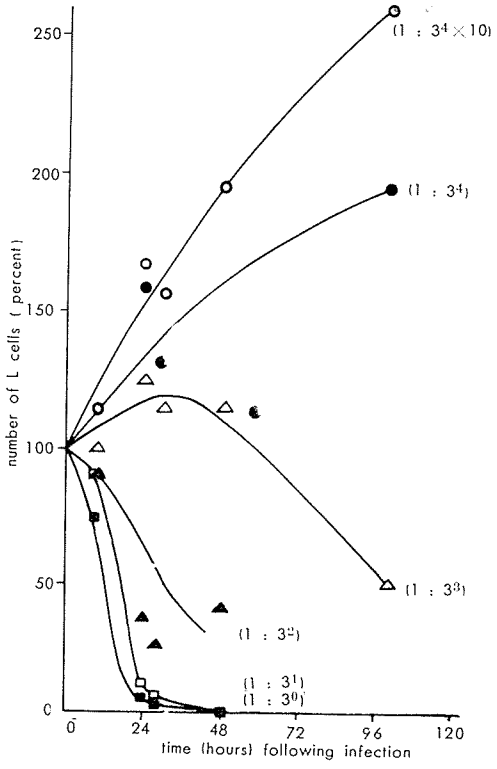

Fig. 6 Cell Response to Infection with the —GCr Miyama Strain of Herpes Simplex Virus

■——■ inoculated with a virus dilution of 1 : 3^0
□——□ inoculated with a virus dilution of 1 : 3^1
▲——▲ inoculated with a virus dilution of 1 : 3^2
△——△ inoculated with a virus dilution of 1 : 3^3
●——● inoculated with a virus dilution of 1 : 3^4
○——○ inoculated with a virus dilution of 1 : 3^{4+10}

The Undiluted original sample contained $1.0\times10^8 TCID_{50}$ per ml. Two ml aliquots of each diluted sample were inoculated into bottles.

$TCID_{50}$ was inoculated into a bottle, which had been seeded with 1.6×10^6 cells just before virus inoculation. In this series unadsorbed virus was not discarded and nor were cells washed. Subsequently, the supernatant fluid of samples of these three series of cultures were titrated at intervals. Results are shown in Fig. 7. Only in the cultures of the third series did cytopathic changes finally appear. Two and four day old cultures released virus for 2 weeks, the former always having a slightly higher titer.

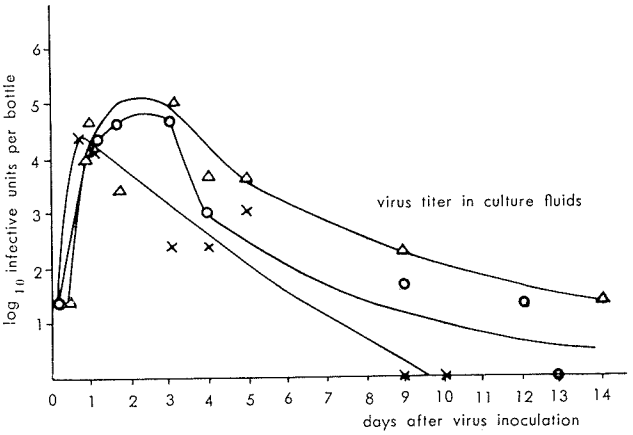

Fig. 7 The Growth of —GCr Miyama Strain on L cell Monolayers

DISCUSSION

Studies on the growth of herpes simplex virus have been made by many investigators using various kinds of host cells and virus strains. Using L cells low viral yields have been reported by some authors, while some host cells produced high virus titers (Scherer, 1953; Kaplan, 1957; Powell, 1959; Hoggan, 1959; Nii *et al.*, 1961; Scott *et al.*, 1961). Our experiments have shown that Earle's L cells infected with the Miyama strain of herpes simplex virus consistently produced lower titers of virus than FL cells. This phenomenon may be generally described by the word "susceptibility". This work is an analysis of this problem at a cellular level.

In a system of FL cells and the —GCr Miyama strain of herpes simplex virus it has already been reported that the number of infectious virus particles expected from a count of inclusion bearing cells detected between 8 and 9 hours after infection coincided with the viral titer counted by the microplaque method. Due to early disruption of infected L cells, the time of recycling could not been ascertained and due to difficulties in the plaque counting titration method in this cell line, a comparison between the numbers of virus particles measured as plaque forming units and as inclusion forming units was impossible. However, in FL and L cell monolayers exposed to the same dose of virus, approximately the same number of inclusion bearing cells appeared. Benporat *et al.* (1961) reported that noninfectious virus particles of pseudorabies produced by Mitomycin C partially retained their ability to induce intranuclear inclusions. Therefore it seems significant that, when the same virus material was used, the number of virus particles effecting inclusions in the two cell lines was found to be equal.

On the other hand FL cells gave a higher value in infective titrations when free virus was used. This may be due to the following two reasons. 1) Of the infected L cells with inclusions in the nuclei, only some form infective centers so

that many infected cells give rise to abortive infections. 2) Infection of L cell monolayers may be arrested due to the low viral yield.

A similar phenomenon was reported by Roizman, who showed by simultaneous titration, that free MP virus of herpes simplex virus induced nearly twenty times more polykaryocytes in cultures of HEp-2 cells than in cultures of mouse embryo cells (Roizman, 1962).

In our experiments performed to know the number of infective centers formed by L cells, virus samples of a titer that did not cause multiple infection were used and it was shown that infected cells often gave rise to abortive infections. If they had been infected with a high multiplicity of virus particles, they might have released much extracellular virus. This possibility cannot been ruled out.

The significance of the eosinophilic substance of herpetic inclusions is unknown. Inclusion formation not accompanied by a significant dose of infective virus particles was also reported by Reissig *et al.* (1961). The latter authors investigated the effect of 5-Fluorouracil on pseudorabies virus growth in cells and observed morphological changes. Our methods differed from those of the above authors, though our results may be related to theirs. Less dense eosinophilic masses were seen in infected L cells than in FL cells, although this may be related to a difference in the host cells used.

In our earlier paper we reported the serial transfer of the original virus population of the Miyama strain of herpes simplex virus on L cells for half a year and it was pointed out that these cells had a lower susceptibility to infection, than FL cells. One difference in experimental conditions used in the previous experiments and the present ones is in the capacity of the culture bottles used. 200 ml bottles were used in previous work and 50 ml ones in the present work. The latter caused a slightly earlier drop of pH than the former and this delicate physiological conditions of the cultures may affect virus growth on L cells.

Different virus growth kinetics in different host cells and different virus strains may affect the results of experiments with chemical and physical agents. Therefore it is important to see whether complete virus growth occurs in a given experimental system.

The virus latency of herpes simplex virus *in vivo* is a very interesting problem. In L cells infected with a low virus input of the —GCr Miyama strain of herpes virus, virus sometimes disappeared after infection, while cells proliferated and sometimes subcultures could be made. The latter cultures, however, are not resistant to a second dose with a high titer of virus and therefore the phenomenon does not represent either a lysogenic state or a state of viral autointerference.

The earlier disruption of L cells than of FL cells after infection, is a more biological problem. This cytological difference between the two cell lines is too complex to explain. Experiments are now in progress using PS cells.

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