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Title	Persistent Infection with Herpes Simplex Virus in vitro I. Establishment and Characteristics of Persistent Herpes Simplex Virus Infection in Earle's L Cells
Author(s)	Nii, Shiro
Citation	Biken journal : journal of Research Institute for Microbial Diseases. 1969, 12(2), p. 45-67
Version Type	VoR
URL	https://doi.org/10.18910/82830
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BIKEN JOURNAL Vol. 12, 45-67, 1969

PERSISTENT INFECTION WITH HERPES SIMPLEX VIRUS *IN VITRO* 1. ESTABLISHMENT AND CHARACTERISTICS OF PERSISTENT HERPES SIMPLEX VIRUS INFECTION IN EARLE'S L CELLS[1,2]

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(Received March 6, 1969)

SUMMARY Persistent infections of herpes simplex virus in Earle's L cells were established and have been maintained for more than four years. The characteristics of the persistent infections were as follows. (1) A balanced state of virus and cell growth in which atypical cycles of rise and fall in virus infectivity were shown was the most common feature. Occasionally increased virus synthesis and great cell destruction followed by regrowth of cells occurred in the cultures. (2) Histological and fluorescent antibody studies on cultures with persistent infection revealed that these cultures were composed of infected and non-infected cells. (3) Addition of antibody to the culture medium is not necessary to maintain this persistency. (4) Persistent infection could be maintained without subdividing cultures and subcultures were made with cells suspended in the fluid phase of the cultures. (5) No interference of culture fluids of persistently infected cultures with plaque formation of vaccinia virus has so far been detected.

INTRODUCTION

One of the most intriguing phenomena among various host-virus interactions is that herpes simplex virus in man persists during intervals between recurrences in the presence of a high level of circulating antibody against the agent.

In studies on the mechanism by which herpes simplex virus persists during the latent period various tissue culture models have been used. First, persistency of herpes infection *in vitro* was established by adding anti-herpes antibody to culture media (Wheeler and Canby, 1959; Fernandetz, 1960; Hinze and Walker, 1961; Szanto, 1963). Second, from examination of an antibody-free system, the role of interferon was considered as one factor to initiate and maintain persistent herpes virus infections

1 This investigation was aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

2 This work was presented in part at the 15th Annual Meeting of the Society of Japanese Virologists in Chiba, on Octber 31, 1967.

3 Part of this work was carried out while the author was a Postdoctoral Fellow in The Department of Microbiology, College of Physicians and Surgeons of Columbia University, New York.

(Glasgow and Habel, 1963; Waddell and Sigel, 1966) and reduced temperature was essential for its establishment (Coleman and Jawetz, 1961). Third, with Chinese hamster cell lines Hampar and Copeland (1965) established persistent infections under routine tissue culture conditions. Their systems were quite unique compared with previous models where various special conditions were more or less needed to initiate and maintain the persistency. The persistent infections by the latter authors were characterized by cycles of cell destruction, with virus synthesis followed by regrowth of the cells.

In a previous paper concerning one aspect of interactions between the -GCr Miyama strain of herpes simplex virus and L cells (Nii and Kamahora, 1962) it was shown that with a low virus multiplicity of virus infection some infected L cells could release extracellular infective virus while other cells showed abortive infection resulting in inclusions in the nuclei. Thus after a limited extent of virus growth the culture recovered from infection. With the same cell line Blackmore and Morgan (1967) reported persistency of herpes infection for approximately 50 days and suggested that a viral-interfering factor (VIF) appeared to be responsible for the carrier state. Independently of this work similar persistent infections were established with a system of -GCr Miyama strain and L cells and have been maintained for a period of more than four years. This report is on the establishment and characteristics of this persistency of herpes infection.

MATERIALS AND METHODS

1. *Virus*

The -GCr variant of the Miyama strain of herpes simplex virus was used for establishment of persistent infection with herpes virus (Nii and Kamahora, 1961). The IHD strain of vaccinia virus which has been kept in this laboratory (Nii, 1959) was grown in Earle's L cells and used as a challenge virus in tests on interference activity.

2. *Cells*

Earle's L cell line was obtained from the tissue culture laboratory of Dr. H. M. Rose, Columbia University. The cells were cultured with Eagle's Minimum Essential Medium (MEM) supplemented with 10% calf serum and maintained by a routine tissue culture technique.

L cells persistently infected with herpes were cultivated in 30 ml Falkon plastic bottles or 50 ml square glass bottles and subcultures were made with suspended cells in the fluid phase of the persistently infected cultures by either one of the following two methods. (1) The culture fluid with the suspended cells was introduced into new flasks with or without addition of an appropriate amount of fresh medium, and on the following day when the living cells had become attached to the bottom of the flask the culture fluid was replaced with fresh medium. (2) The suspended cells were sedimented by low speed centrifugation, resuspended in fresh medium and introduced into new flasks.

3. *Infectivity titration*

Infectivity titrations were carried out in FL cells by the 50% endpoint method in tube titrations ($TCID_{50}$). Monolayer cultures were prepared in a series of test tubes containing 2 to 3×10^5 FL cells. For virus inoculation, the nutrient media were removed from the test tubes and 0.1 ml samples of tenfold dilutions of virus were added to the tubes. Three or four test tubes were used for each dilution. After a 2 hr adsorption period 1.5 ml of MEM supplemented with 5% calf serum was added. Fifty percent cytopathic doses were calculated by the Reed and Muench method from the number of test tubes showing cytopathic changes after one week.

4. *Grade of CPE and number of cells in persistently infected cultures*

CPE was differentiated into five main grades, 0, +1, +2, +3 and +4, and intermediates between these were noted. These grades were as follows:

- 0 .... No CPE was observed.
- +1 .... A limited extent of CPE was found in a few restricted areas of the culture.
- +2 .... Approximately half the cells in the culture appeared to be infected.
- +3 .... Almost all the cells were affected.
- +4 .... Total destruction of cells occurred and many disrupted cells were observed. Few if any normal looking

cells were found.

The number of cells in the culture was estimated microscopically and classified into four main grades, 0, +1, +2 and +3, and intermediates between these as follows:

0 .... Few if any healthy cells were observed in the culture.

+1 .... Colonial cell growth was observed in limited areas.

+2 .... Approximately half the bottom of the flask was covered with cells.

+3 .... A complete, or nearly complete monolayer sheet was formed.

5. *Test of interference activity*

To assay interfering activity of culture fluids of cells with persistent herpes infection on the growth of virus, a plaque reduction test with the IHD strain of vaccinia virus was performed as follows. The culture fluids of L cells with persistent infection were centrifuged at 3000 rev/min for 10 min. The supernatant fluids were removed and inoculated onto Earle's L cell monolayers in 50 ml square bottles. As controls parallel monolayers were inoculated with similarly treated supernatant fluids of normal uninfected L cell cultures. After appropriate incubation periods the supernatant fluids were removed from the monolayers and a countable PFU (i.e. 50 to 200) of the IHD strain of vaccinia virus was inoculated onto each monolayer. After adsorption for 1 hr the inocula were replaced by maintenance media and plaques were counted 72 hr later.

6. *Fluorescent antibody technique*

Herpes simplex fluorescent antibody was kindly supplied by Dr. Hayashi of the Institute of Medical Sciences, Tokyo University through Dr. Aoyama of the same institute. Briefly this had been made as follows; Antiserum for herpes simplex virus was made from a rabbit infected corneally with a newly isolated strain of herpes simplex virus. The antibody was conjugated with FITC using the routine method in Dr. Kawamura's laboratory and the conjugated antibody was adsorbed with human liver powder.

The staining procedures used with the antibody were as follows. Cells on a cover slip were washed twice with PBS, air dried and fixed with acetone at room temperature for 10 min. After evaporation of the acetone, the cells were washed once with PBS and covered with antibody at 37 C for 30 min in humid conditions. The stained preparation was washed well with PBS in a dish and examined and photographed through a Nikon microscope with a Chiyoda UV-light source.

7. *Histological observation*

Cells from cultures with persistent herpes infection on coverslips were washed once with PBS, fixed with Bouin's fixative and stained with hematoxylin and eosin. Histological preparations were observed with a Nikon light microscope.

8. *Electron microscopy*

Suspended cells in the fluid phase of persistently infected cultures were sedimented by centrifugation at 1000 rev/min for 10 min. The resulting pellet was fixed for 1 hr in 1% glutaraldehyde, washed well, fixed for 30 min in 1% osmium tetroxide, dehydrated and embedded in epoxy resin. Sections were made with glass knives in an LKB microtome and stained with uranylacetate and lead citrate. A hitachi 11 B electron microscope was used for observation.

RESULTS

1. *Serial viral passages and establishment of persistent infections in Earle's L cells*

An L cell monolayer with about 6×10^6 cells in a 200 ml square bottle was inoculated with 3 ml of approximately 10^8 $TCID_{50}$ of the -GCr Miyama strain of herpes simplex virus. After adsorption for 1 hr the inoculum was replaced by maintenance medium. Thereafter, serial viral passages were performed with the supernatant fluids of the culture media when the CPE in the culture at each generation of viral passages was greatest (Table 1). A series of viral passages with the same host-virus system has already been reported (Nii and Kamahora, 1961) and the type of CPE seen and the viral yields found in this experiment were the same as those reported earlier.

After eight consecutive viral passages it was noticed that some of the infected cultures in earlier generations seemed to have recovered from viral affection. In the cultures of the second and third passage (L_{II} and L_{III} in Table 1 and Fig. 1) regrowth of cells had occurred

TABLE 1. *Serial passages of the -GCr Miyama strain of herpes simplex virus in Earle's L cells*

Dates of the first virus inoculation[a] and the following virus passages	Generation of serial virus passages[b]	Virus titer[c]
Nov. 5 1964	L_I	$10^{6.8}$
6	L_{II}	$10^{5.0}$
8	L_{III}	—[d]
11	L_{IV}	$10^{5.8}$
13	L_V	—
15	L_{VI}	—
17	L_{VII}	—
19	L_{VIII}	—

a: Three ml of approximately 10^8 $TCID_{50}$ of the -GCr Miyama strain of herpes simplex virus were inoculated onto an L cell monolayer grown in a 200 ml square bottle and after one hour's adsorption the inoculum was replaced with maintenance medium. This culture was designated as L_1.
b: Serial virus passages were performed as follows; when CPE in the culture at each generation of viral passage was greatest, the culture fluid was removed and centrifuged at 3000 rev/min for 10 min. and 2–3 ml of the supernatant fluid was inoculated into a fresh culture for the next generation of viral passage. After 1–2 hour's absorption the inoculum was replaced with maintenance medium.
c: As described in the above footnote, the culture fluid was removed when CPE in the culture at each generation of viral passage was greatest and titrated. The titer is expressed as $TCID_{50}$/ml.
d: not tested.

and colony-like areas of variable size were observed. The culture fluid of L_{IIa} in which no CPE was detected was removed twice for infectivity titration, i.e. once, three weeks after the viral transfer to the culture and again, two days later. No infectivity was found in the fluid and the culture was found to be free from virus infection. Further cultivation of L_{IIa} was stopped (Fig. 2). In another culture at the same generation of viral passage, i.e. L_{IIb}, CPE also became undetectable although it had been great soon after passage of virus to the culture. Thirty four days after L_{IIb} was prepared the culture medium was removed for infectivity titration to see whether any virus still remained in the culture. This culture was divided into three new cultures. Titration revealed no infectious virus, and no CPE was observed on further cultivation of the latter cultures. This phenomenon of recovery from infection of L cells after limited growth of virus has already been reported (Nii and Kamahora, 1962; Blackmore and Morgan, 1967).

To see if there was any difference in the susceptibility of the cells to herpes simplex virus after recovery from infection two of the three cultures described above were challenged with further virus. One was inoculated with about 10^5TCID_{50} of FL cell passaged virus of the -GCr Miyama strain and the other with approximately the same dose of L cell passaged virus. CPE definitely occurred in both cultures, showing that after recovery the cells had no resistance to a second challenge of virus.

On the other hand cell regrowth accompanied with a moderate extent of CPE occurred in two cultures used for the third viral passage (L_{III}). After 17 days viral passage these cultures were divided respectively into two and three cultures (Fig. 1). The resulting five cultures,

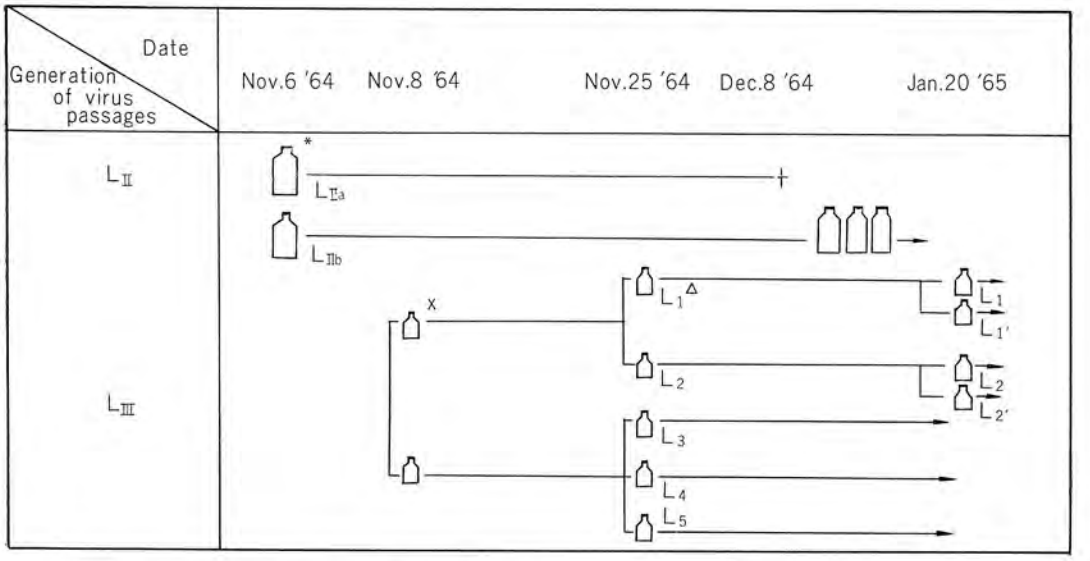


FIGURE 1. *Establishment and maintenance of cultures with persistent herpes infection.*

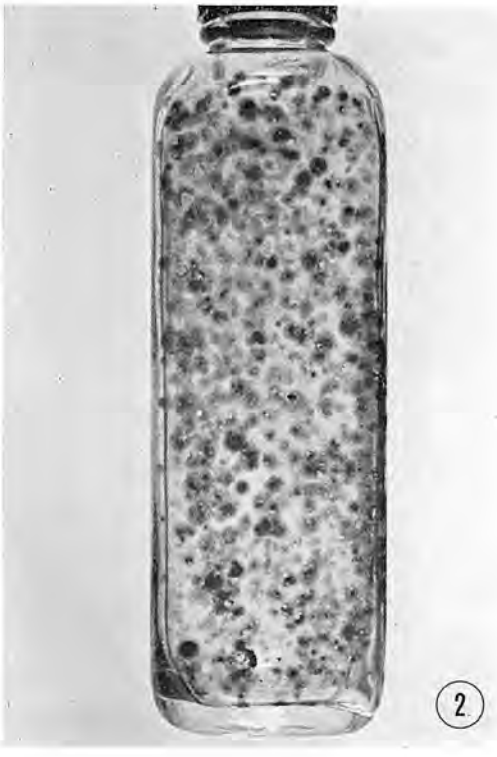


FIGURE 2. *Colonial regrowth of L cells in a* 200 *ml square bottle* (L_{IIa}).

Two L cell monolayers were infected for the second viral passage and in a few days an intense CPE occurred. Later surviving cells grew in both cultures. Thirty two days after virus inoculation one culture, L_{IIa} *was fixed with methanol and stained with Giema's solution.* ×*0.55*

designated as L_1, L_2, L_3, L_4 and L_5, were cultivated in growth medium instead of maintenance medium. Fifty six days after subculture two new cultures, L_1' and L_2', were made by suspending cells in the culture fluids of L_1 and L_2. Thus seven cultures infected with herpes were prepared.

2. *Persistence of herpes simplex virus in Earle's L cells*

1) Maintenance of L cell cultures infected with herpes

To see how long herpes simplex virus persisted in the above seven L cell cultures infectivity was titrated. At intervals from December, 1964 to June, 1965 samples of the culture fluids were titrated (Fig. 3). Viral infectivity was detected in all titrations except for one sample of L_5 culture in which no living cells could be detected microscopically at the time when the culture fluid was removed for titration. The titers mostly ranged from $10^{2.5}$ to $10^{6.0}$ $TCID_{50}$/ml, mainly near $10^{4.5}$ $TCID_{50}$/ml. Even after the viral titer in the culture fluid of L_5 had dropped to the level of no infectious dose, persistent infection continued with subsequent regrowth of the cells and reappearance of CPE.

One characteristic of the persistent infections in Earle's L cells is cycles of rise and fall in virus infectivity accompanied with cell destruction followed by regrowth of cells, as reported

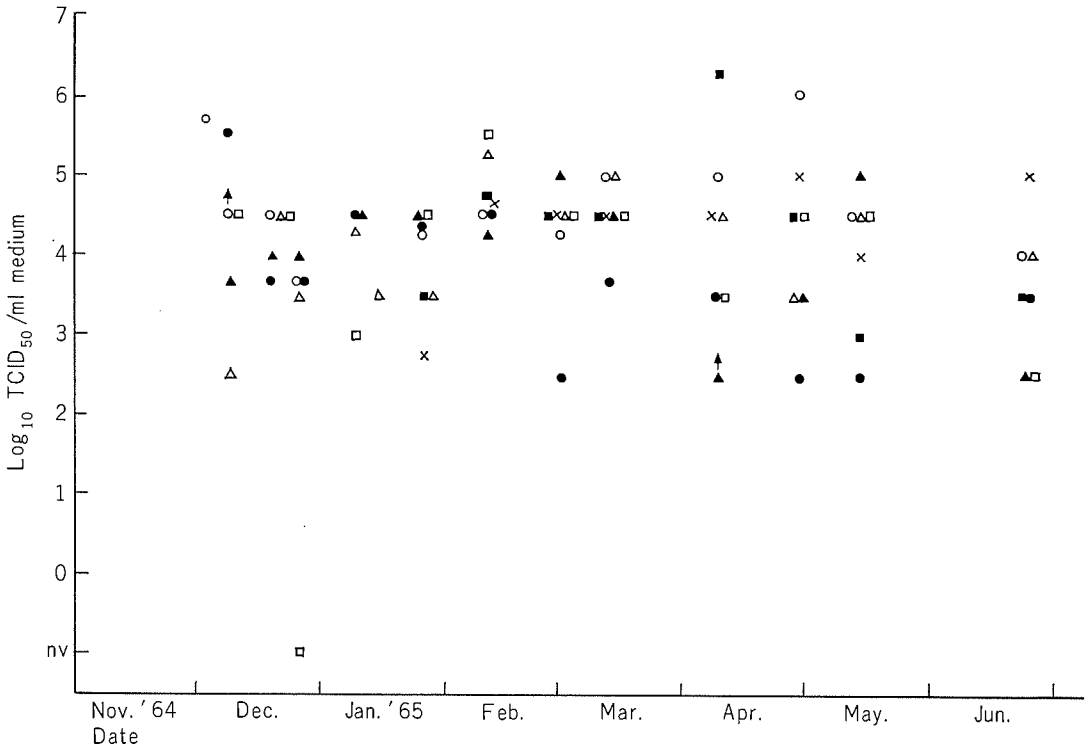


FIGURE 3. *Persistence of herpes simplex virus in Earle's L cells.*
Infectivity titers of culture fluids from 7 cultures with persistent herpes infection;
○: L_1, ●: L_2, △: L_3, ▲: L_4, □: L_5, ■: L_1', ×: L_2'.

by Hampar and Copeland (1965) with Chinese hamster cell lines infected with the same agent. However, the cycles in the L cell cultures varied in duration and in the extent of CPE.

To maintain this persistency no special conditions such as addition of antibody to the medium or cultivation of cells at low temperature was necessary. However care was taken over changing the medium. When the CPE in cultures with persistent infection was most advanced or while only a few cells survived after intense cell destruction, one fourth to one third of the total volume of the culture medium was changed for fresh medium. A similar schedule was used by Waddell and Siegel (1966). Although this care in change of medium may be not essential to maintain persistent infection, it was taken to protect the cells from total destruction due to increased virus growth in fresh medium and to maintain constant culture condition, especially to prevent the culture medium from increasing to above the physiologically optimum pH. This caution is not now necessary.

In February 1966 six viral samples from cultures L_1, L_1', L_2, L_2', L_3 and L_4 were titrated and the culture fluids of L_1, L_1', L_1'', L_2, L_2' and L_4' were also titrated occasionally from July to October 1966 (Fig. 4). L_1'' and L_4' are subcultures of cells in the fluid phase of L_1' and L_4, respectively.

The above data indicate that persistent in-

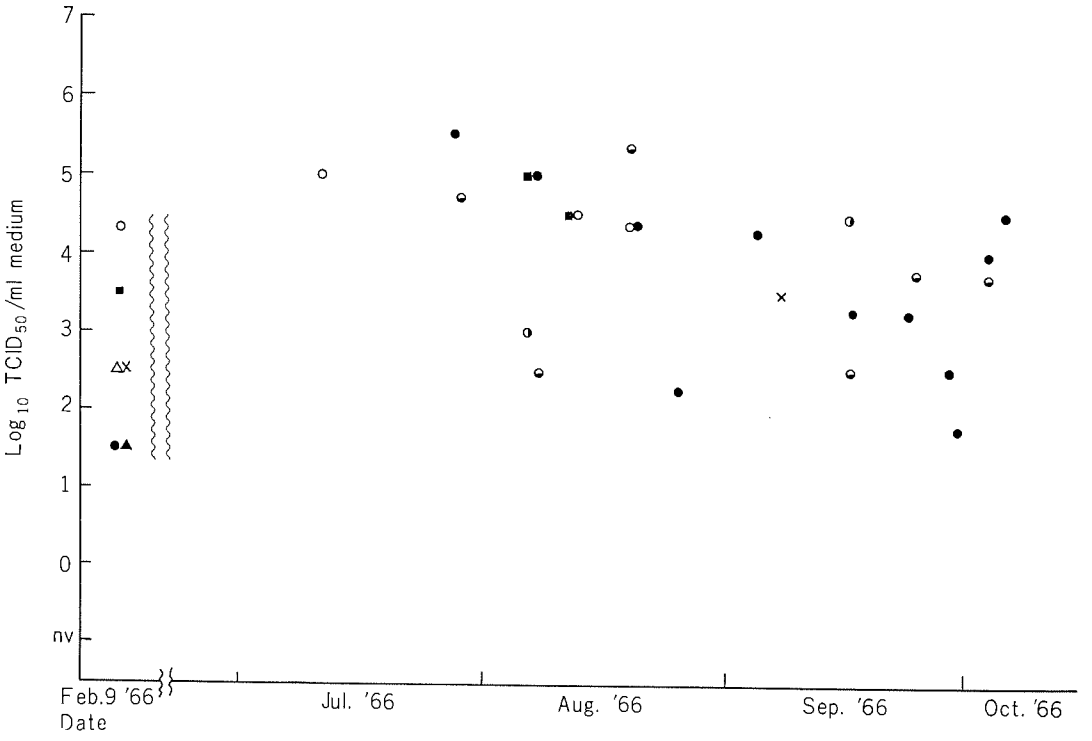


FIGURE 4. *Persistence of herpes simplex virus in Earle's L cells.*
Infectivity titers of culture fluids from 8 cultures with presistent herpes infection;
○: L_1, ●: L_2, △: L_3, ▲: L_4, ■: $L_{1'}$, ×: $L_{2'}$, ◒: $L_{4'}$, ◑: $L_{1''}$.

fections of herpes simplex virus in Earle's L cells are fairly stable.

2) Characteristics of persistent herpes infection in Earle's L cells

To learn more about the characteristics of this persistent infection extensive titration of infectivity in two cultures L_1 and L_2 was done for eleven months from November, 1966 to October, 1967. In this period the culture fluid of each culture was titrated several times per month except in March, 1967 when titrations were done at two day intervals. The titer was correlated with the extent of CPE as well as the number of cells in the culture at the time when the viral sample was removed for infectivity titration (Figs. 5 and 6). Periodic increase and decrease in infectivity titers were comparatively clear in culture L_1 until June, 1967. Generally, while cells were increasing in number the CPE observed in the culture seemed to be arrested at a low level. This tendency was seen in culture L_1 for one month from the middle of February, 1967 and in culture L_2 for two months in November and December, 1966 and for one month each in July and September, 1967. When the infective titer was minimal between cycles, a fair number of cells were usually found or a few cells were just on the point of active regrowth.

During this eleven month period the greatest CPE in the both cultures was observed in February, 1967, accompanied by a typical cycle of infectivity titer and almost all the cells were killed, so that no living cells could be detected microscopically. With culture L_2 rise and fall in infective titer, simultaneously with increase and decrease of CPE and decrease in cell number were more typical and a thirty six days period from January 21 to February 26 in 1967 was needed for one cycle (Feg. 6). In culture L_1 a complete cycle took about 60 days, corresponding to the two months of January and February, 1967 (Fig. 5). Thus the time needed for one cycle in infective titer was much longer than the period of about 16 days reported by Hampar and Copeland (1965).

To see whether more frequent change of medium modified the pattern of the cycle of infectivity titer and especially whether it shortened the time needed for one cycle by causing faster spread of virus infection and accelerated regrowth of cells, medium was changed at two day intervals from the end of the above typical cycles and infectivity titrations were continued. As shown in Figs. 5 and 6, with culture L_2 a new cycle appeared which lasted 30 days while with culture L_1 there were two small cycles lasting 15 days each. These new cycles in the two cultures, however, were not typical. The maximum titer was not high and there was only a small difference between the maximum and minimum titers in the cycles. Moreover these cycling patterns of infectivity were not accompanied by severe CPE. Thus, typical cycles of shorter duration did not occur in the system of Earle's L cells and Miyama strain herpes simplex virus.

The above data indicate that in the present system of virus and host cell the cycling patterns of infective titers with periodic destruction and regrowth of cells were rather irregular and occasionally were not clear for long periods and that when a typical cycle appeared its completion took one to two months.

3) Mode of viral persistence in fresh subcultures

It has frequently been observed that in freshly prepared cultures of persistent infection cycling patterns of CPE and viral infectivity titer are very clear while in old cultures they are not.

This phenomenon was also shown in the following experiment. Suspended cells in the fluid phase of culture L_2 were sedimented and resuspended in fresh medium supplemented with 5% antiserum. The cell suspension containing about 2×10^5 cells was introduced into a 30 ml Falkon bottle to form one fresh subculture and two weeks later antibody was freed from the culture. Within another one week a complete monolayer sheet was formed and a moderate number of cells began to be suspended in the fluid phase of the culture. Three weeks after preparation of the first sub-

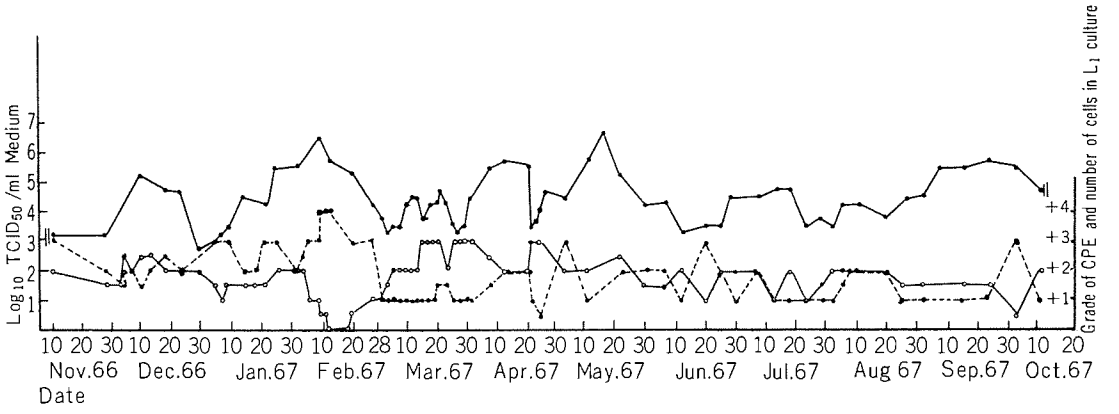


FIGURE 5. *Persistent infection of herpes simplex virus in L cell culture L_1.*
-●——●-: *infective titer of culture fluid from culture L_1.*
-●-----●-: *extent of CPE. This was graded microscopically as described in Materials and Methods.*
-○——○-: *estimated number of cells. This was graded microscopically as described in Materials and Methods.*

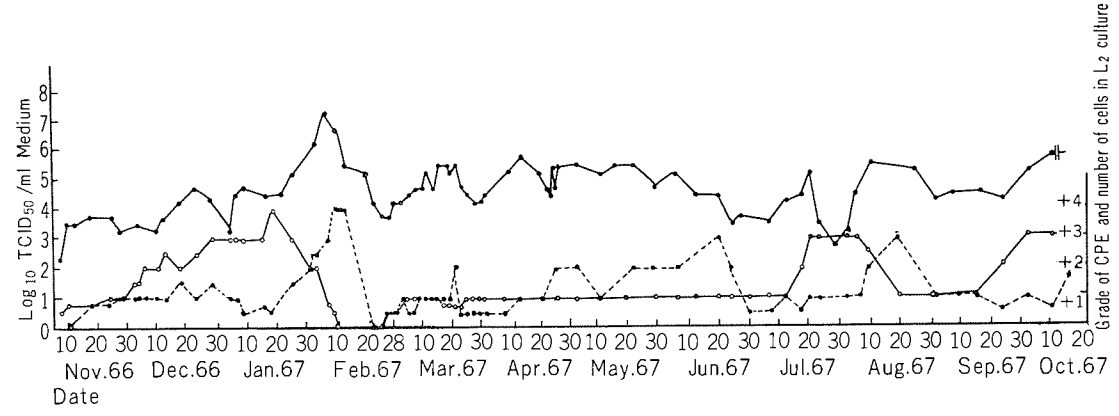


FIGURE 6. *Persistent infection of herpes simplex virus in L cell culture L_2.*
-●——●-: *infective titer of culture fluid from culture L_2.*
-●-----●-: *extent of CPE. This was graded microscopically as described in Materials and Methods.*
-○——○-: *estimated number of cells. This was graded microscopically as described in Materials and Methods.*

culture, a second one was made from cells in the fluid phase of the first; i.e. a cell suspension containing about 4×10^5 cells derived from the first culture was put into a new bottle. Thus, two successive subcultures were prepared. Fig. 7 shows the patterns of infective titer of the culture fluids obtained from the two cultures in which an initial latent period of virus infectivity was followed with one clear cycle in each culture, the cycle in the second subculture being especially clear. In the second culture no infective virus was detected for about forty days after the cycle while in the first subculture a low viral titer was maintained continuously. In relation to this no healthy looking cells were observed microscopically for about one month in the second culture or for about two weeks in the first culture.

For a few days before and when the viral titer started to rise in the two subcultures, no difference was observed microscopically between the two cultures in the number of cells in each culture or in the morphological appearance of the cells. Nevertheless in the second subculture a more clear-cut cycle of infectivity was seen and more complete destruction of cells occurred and this may indicate that virus growth was influenced by delicate cellular

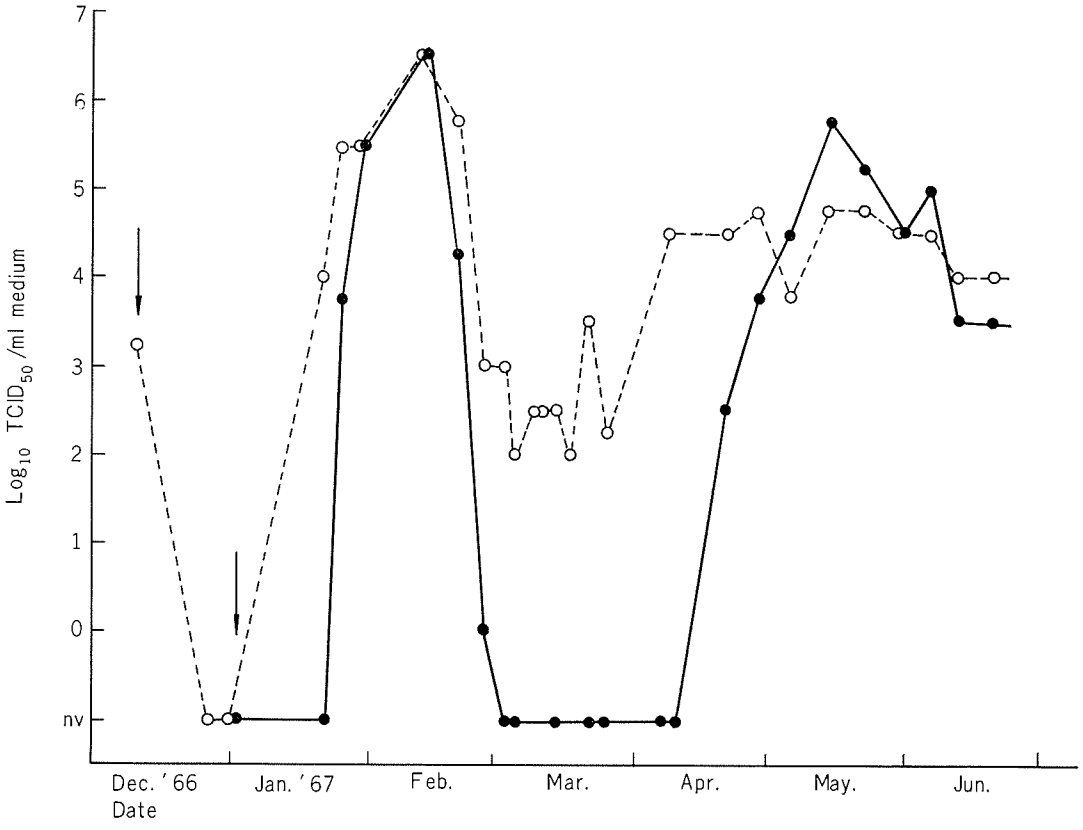


FIGURE 7. *Persistence of herpes simplex virus in two successive subcultures.*
Two successive subcultures were made on the days indicated by arrows.
-O-----O-: *infective titers of culture fluid from the first subculture.*
-●-----●-: *infective titer of culture fluid from the second subculture.*

conditions such as the age of cultures.

3. *Morphological observations*

1) Cytopathic effect

In cultures with persistent herpes infection cytopathic changes were more or less obvious except in cultures in which cells were growing so actively that there were many cells suspended in the medium.

Figs. 8 and 9 show the appearance of cells in cultures L_1 and L_2. Spindle shaped and normally looking cells were interspaced between big cell masses, and very fine granules were distributed in vacant areas on the wall of the bottle. These cells had been cultivated for two years and nine months in the same containers before the pictures were taken and therefore it was supposed that the granules represented disrupted cell debris which had accumulated even though the medium was changed frequently.

Fig. 10 shows the cytopathic changes in a fresh 35 day old subculture of L_2 at the time of the first wave of severe infection. After the greatest degree of CPE most infected cells had become detached from the bottle wall but some disrupted cells and cell debris still remained. A few normal looking cells were also seen and these might survive and multiply to form a colonylike area which would finally fuse with other similar areas resulting in formation of a new monolayer sheet. The background in this picture which corresponds to the wall of the container was comparatively clean indicating that accumulation of fine granules was not yet remarkable.

2) Intranuclear inclusion bodies

Cultures presistently infected with herpes prepared on coverslips in Leighton tubes were examined histologically. Intranuclear inclusion bodies in various developmental stages were seen. In Figs. 11 and 12 some infected cells have clear intranuclear inclusion bodies with a halo surrounded with marginated chromatin. Some round cells showing pyknosis seemed to be about to disruption (Fig. 12).

3) Fluorescent antibody study

Cells with persistent infection grown on coverslips were examined with the fluorescent antibody technique. Fig. 13 shows a small focal area of fluorescent cells. Similar fluorescent foci were detected even in cultures in which CPE was not clearly observed. Cytopathic foci laced with many fluorescent cells such as that shown in Fig. 14 were a common feature of most cultures with persistent infection. In cultures showing intense cytopathic changes many round cells containing herpes virus antigens were always observed (Fig. 15).

4) Electron microscopic study

Culture fluids were removed from cultures with persistent infection, in which CPE was marked, and centrifuged. The pellets were embedded for electron microscopic study. In ultrathin sections of the specimens cells with particles characteristic of herpes virus were clearly observed intermingled with disrupted cell debris. Figs. 16 and 17 show many viral particles with capsids located in the nucleus. Almost all the particles had cores of low density and round or ovoid cores were very common. Few rod shaped cores were detected (Fig. 16). In Fig. 19 particles filled with electron dense material as already reported by Nii et al. (1968a) are indicated by arrows. The cytoplasmic portion of an infected cell was lost on advanced cell degeneration and the nucleus was exposed (Fig. 17). A crystal array of viral capsids was rarely observed (Fig. 18). Reduplication of nuclear membranes occurred (Figs. 18 and 19) but very few particles with envelopes were detected. In all the nuclei shown in the above pictures condensation of chromatin was marked.

4. *Test of culture fluids of L-cell cultures with persistent infection for interfering activity on plaque formation of vaccinia virus*

The supernatant of the culture fluids prepared by low speed centrifugation was used directly for assay of interfering activity or after appropriate dilution. L cell monolayers inoculated with the sample were incubated at 37 C

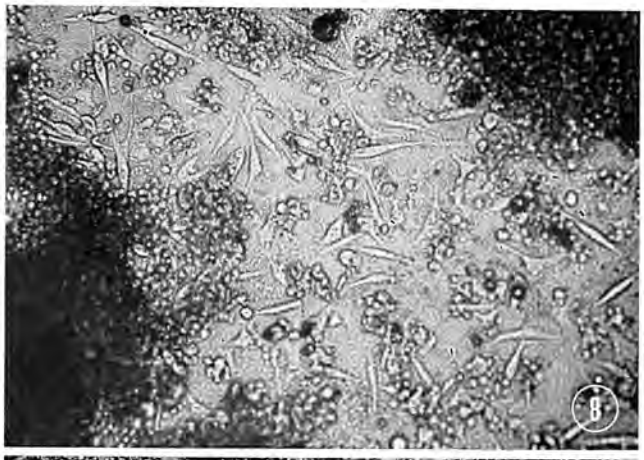


FIGURE 8. *Cytopathic effect in culture L_1 with persistent herpes infection.*

Spindle shaped and normally looking cells interwove between big cell masses, and very fine granules were dispersed throughout the vacant areas. ×120

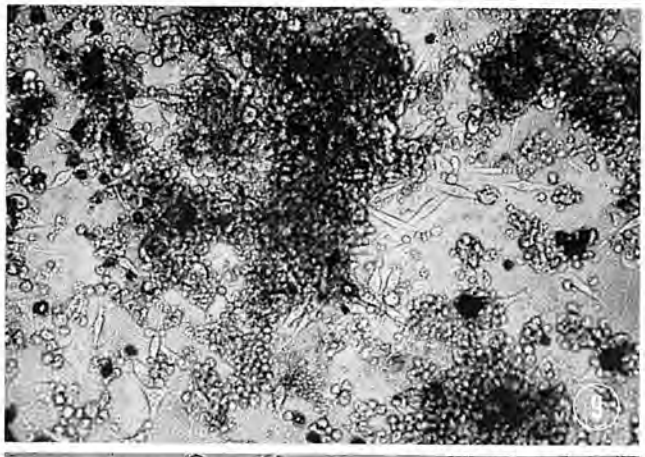


FIGURE 9. *Cytopathic effect seen in culture L_2 with persistent herpes infection.*

Spindle shaped cells seemed to proliferate from large dense masses. Disrupted cells and very fine granules were also seen. ×120

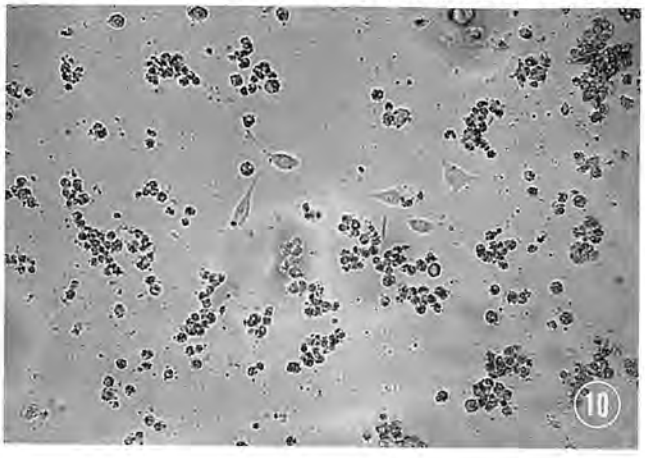
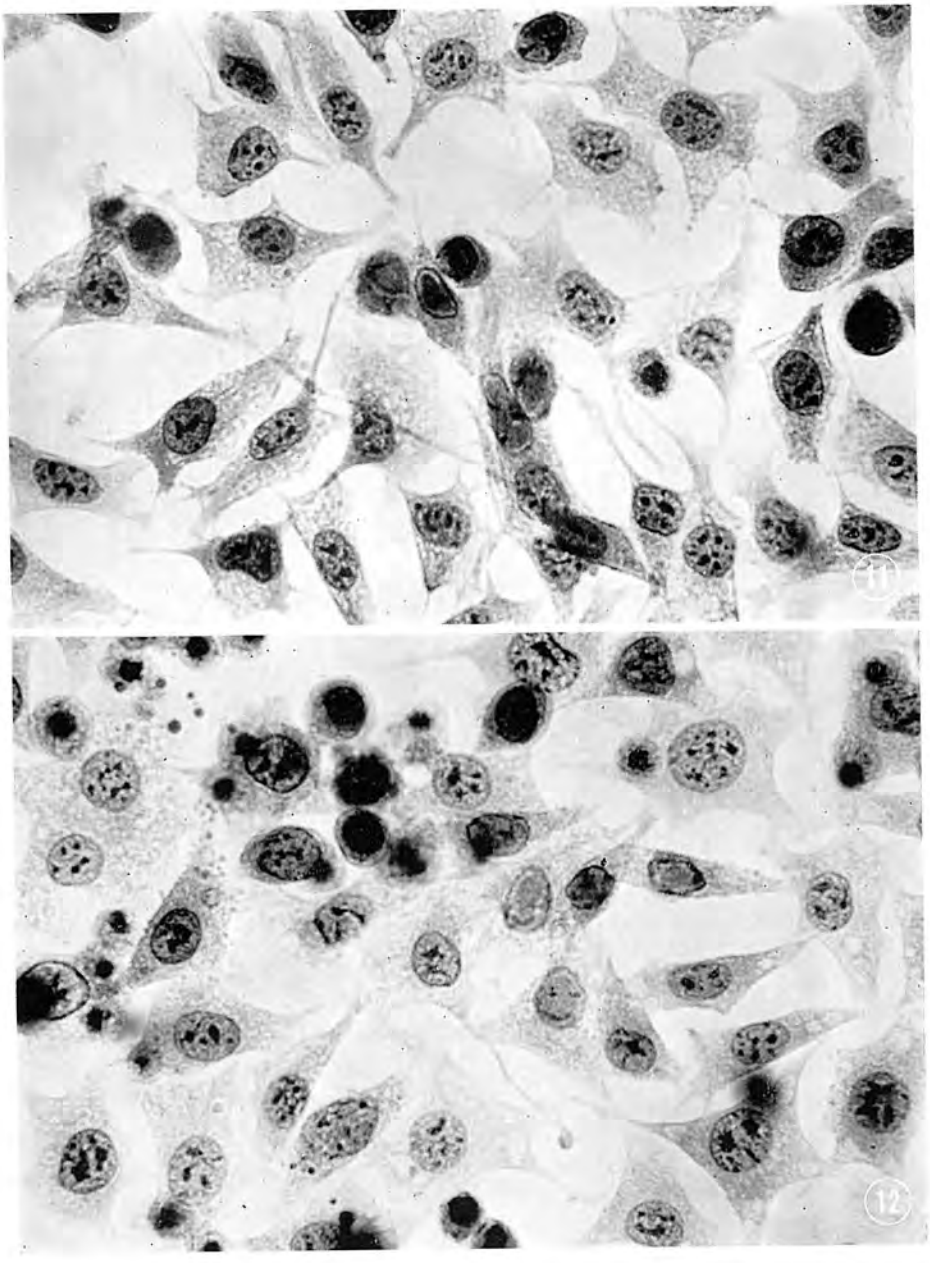
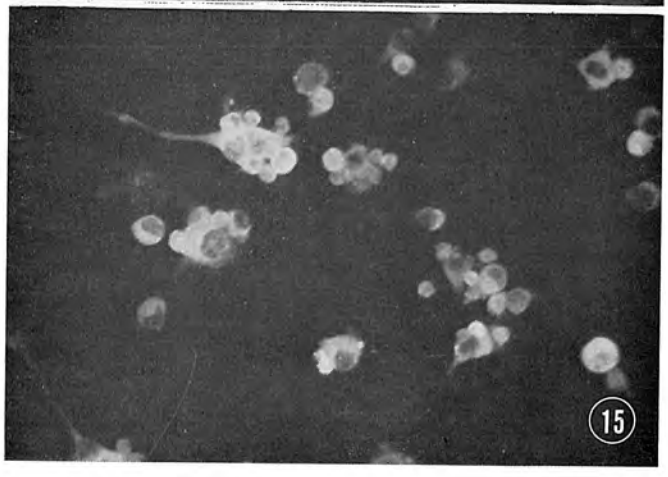


FIGURE 10. *Cytopathic changes in a fresh subculture prepared from L_2.*

A few normal looking cells, some disrupted cells and cell debris were seen. ×120



FIGURES 11 and 12. *Intranuclear inclusion bodies in some cells in cultures with persistent infection. Early stages of development of inclusions between condensed chromatin as well as typical inclusions surrounded by a halo were observed. Several rounded cells showing pyknosis were also seen (Fig. 12). Fixed with Bouin's fluid and stained with hematoxylin and eosin.* ×1200



FIGURES 13, 14 and 15. *Cultures with persistent herpes infection stained with fluorescent antibody.*

A small focus of fluorescent cells is shown in contrast with a dark area of noninfected cells (Fig. 13).

Fig. 14. *shows a cytopathic focus laced with many fluorescent cells.*

The cells undergoing severe CPE always contained much viral antigen (Fig. 15). ×600

TABLE 2. *Test of interference of fluids of L cell cultures with persistent herpes infection on plaque formation of vaccinia virus*

		Samples used for pretreatment[a] of L cell monolayers before a challenge with vaccinia virus		
		Lc [b]	Mc [c]	L_1 JAN4'67[d]
Exp. NO. 1	Number of plaques produced	191 194 171	178 165 177	174 190 189
	Average number of plaques	185	173	181

		Samples used for pretreatment[a] of L cell monolayers before a challenge with vaccinia virus				
		Lc [b]	Mc [c]	L_1 JAN6'67 [e]	L_1 JAN8'67 [e]	L_1 JAN14'67 [e]
Exp. NO. 2	Number of plaques produced	111 72 105	95 81 111	83 132 72	78 110 88	106 96 107
	Average number of plaques	96	97	97	92	103

		Samples used for pretreatment[a] of L cell monolayers before a challenge with vaccinia virus		
		Lc [b]	Mc [c]	L_2 JAN16'67 [f]
Exp. NO. 3	Number of plaques produced	130 133 136	137 143 166 158	187 145 167
	Average number of plaques	133	151	166

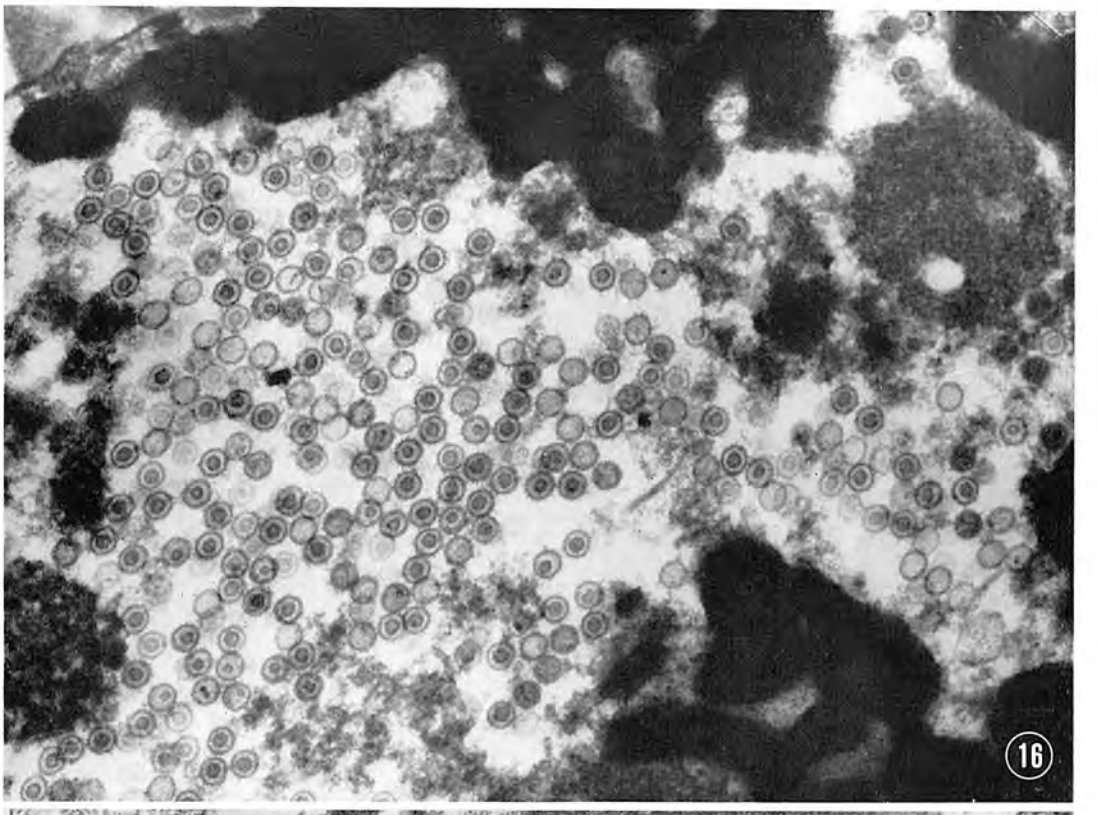
a: L cell monolayers were pretreated with samples for 6, 1.5 and 10 hrs respectively in experiments NO. 1, 2 and 3.
b: Supernanant of culture fluids obtained from non-infected Earle's L cells.
c: Fresh Minimum Essential Medium supplemented with 10 percent calf serum.
d: Supernatant of culter fluid from culture L_1 with persistent herpes infection on Jan. 4 in 1967. It was used without dilution.
e: Supernatants of culture fluids obtained from L_1 respectively on Jan. 6. 8 and 14, 1967. They were used after six fold dilution with fresh Minimum Essential Medium supplemented with 10 percent calf serum.
f: Supernatant of culture fluid obtaied from L_2 on Jan. 16, 1967. It was used after three fold dilution with fresh Minimum Essential Medium supplemented with 10 percent calf serum.

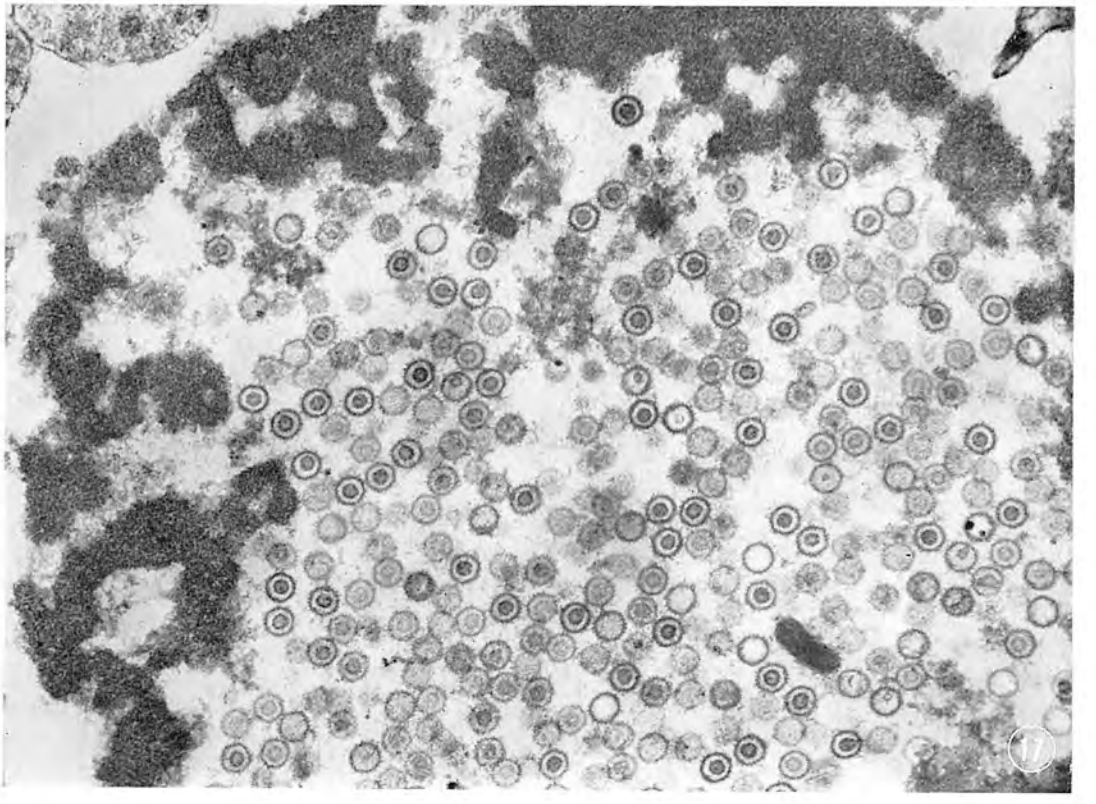
for three different periods; i.e. 1.5, 6 and 10 hr. As controls supernatant fluids of media from non-infected Earle's L cell cultures and fresh media were also inoculated onto similar monolayers and the cultures were incubated for ths same period as the experimental group. The inocula were replaced by viral samples with a countable number of PFU of vaccinia IHD strain and three days later the number of plaques produced was counted. These were clearly observed (Nii, 1959) and distinguished from small comet-like focal lesions formed by the virus of herpes simplex carried in the present cultures of persistent infection. The results are shown in Table 2. In these experiments interfering activity of the supernatant of the cultures with persistent infection on plaque formation of another DNA type virus was not revealed, and the samples did not contain a sufficient infective dose of herpes simplex virus to inhibit formation of plaques of the challenge virus even by a classical interference mechanism.

FIGURES 16 and 17. *Electron microscopy of cells suspended in the fluid phase of cultures with persistent herpes infection.*

Both nuclei shown in the figures contain many viral particles with capsids and cores of low density. Condensation of chromatin is remarkable. The cytoplasmic portion of the cell in Fig. 17 appears to be lost.

Fig. 16 ×37500 *Fig. 17* ×38500

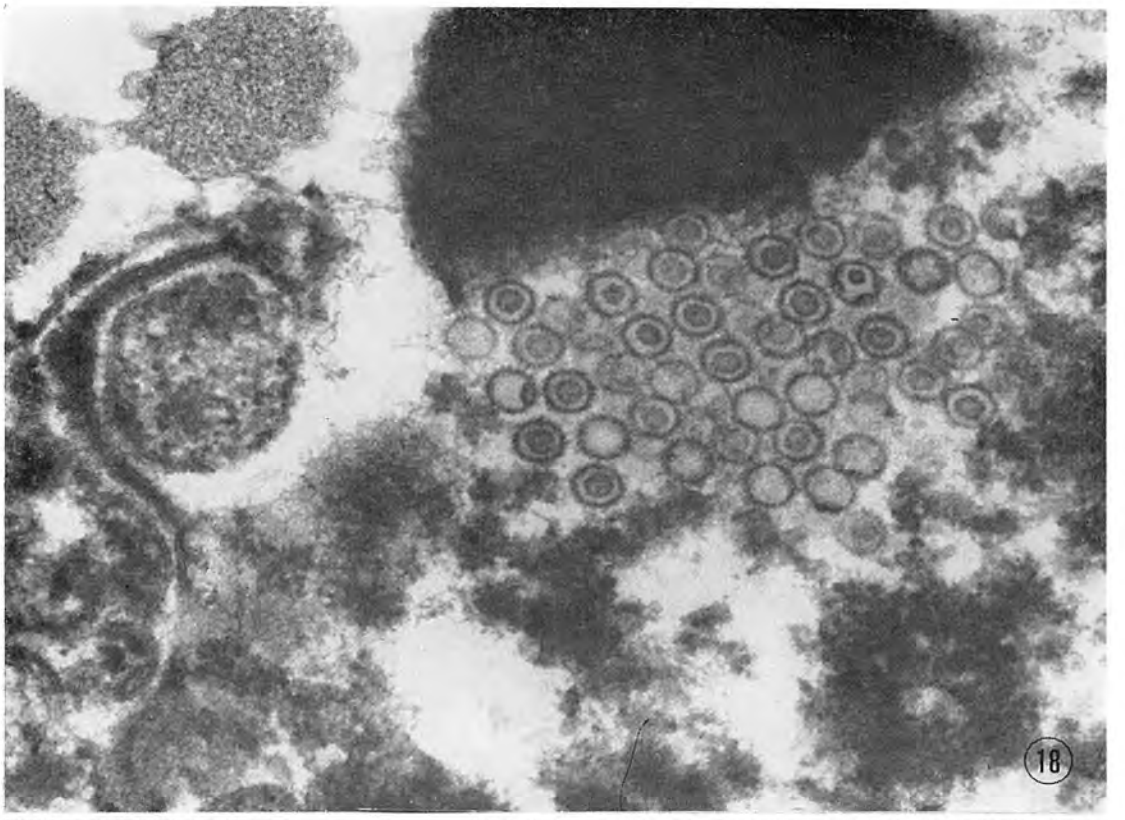

16


17

FIGURES 18 and 19. *Electron microscopy of cells suspended in the fluid phase of cultures with persistent herpes infection.*

Fig. 18 shows a viral crystal which consists of particles with capsids and cores of low density. Reduplication of the nuclear membrane is shown at the left of the picture. *Fig.* 18 *× 69000*

In Fig. 19 two particles containing electron dense material are indicated by arrows. Reduplication of the nuclear membrane and condensation of chromatin are also seen in the picture. *Fig.* 19 *× 16500*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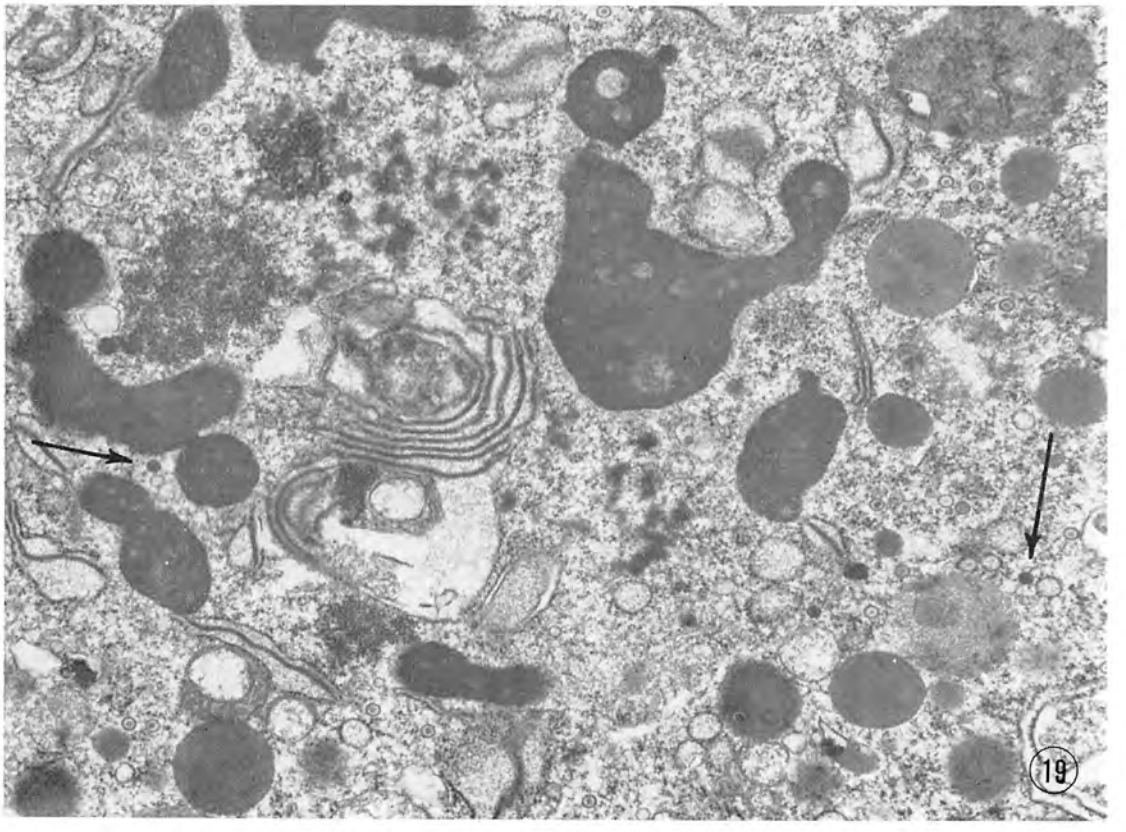
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DISCUSSION

Various kinds of persistent herpes simplex virus infections in vitro have been reported and most of them were established using special conditions such as addition of antibody to the medium or lowering the temperature during cultivation of cells, or co-cultivation of herpes simplex virus with another kind of virus. However, no special conditions were used by Hampar and Copeland (1965) who established persistent infections of virus in Chinese hamster cell lines using a routine tissue culture method or by Blackmore and Morgan (1967) who observed a carrier culture state of the agent for 53 days with an Earle's L cell line. Similarly, with the -GCr Miyama strain of herpes simplex virus and this cell line persistent infections were established and have been maintained for four years and three months.

Primary infection of an L cell culture with -GCr virus, however, did not always lead to establishment of persistent infection. In many cases total cell destruciton occurred and consequently further maintenance of the culture was impossible. Besides, the infected culture occasionally recovered from viral affection and on further cultivation no CPE reappeared. This recovery was very rare in old cultures with persistent infection indicating that in these there was a stable host-virus relationship.

Generally when the most advanced CPE occurred in an L cell monolayer infected with the Miyama strain of herpes simplex virus almost all the cells were killed, so that even single living cells were scarcely detected by microscopic examinations. Eventually the pH of the medium in these cultures rose. Even if a few cells somehow survived infection, some of them would be killed by the unfavorable cultural conditions and only physiologically active cells would survive and participate in formation of a new cell monolayer. Therefore cells may be selected in two ways: by direct virus affection and by subsequent unfavorable environmental conditions. The establishment of persistent infection after primary infection depended on the survival of a few active cells because tissue culture cells were routinely subcultured as a mass cellular population and under these conditions even physiologically inert cells could multiply through many generations. The cells in old cultures with persistent infection seemed to be more active physiologically than routinely passaged Earle's L cells which would partly explains the stability of host-virus interactions of these cultures.

Another cellular factor in establishment of persistent infection with herpes was reported by Hampar and Copeland (1965). These authors studied the susceptibility of two Chinese hamster cell lines to the virus from the time of their initiation through successive subcultures and found that the susceptibility of cells to the cytocidal effects of the agent decreased as the number of cell passages increased and all cultures of the one line (MAL cells) tested subsequent to their 35th passage gave rise to cultures with persistent infections when inoculated at a virus-cell ratio of one or less. They also observed a recovery phenomenon in cultures infected with herpes; when 45th passage MAL cells were infected with virus at a virus-cell ratio of 10, the cells recovered completely from virus infection.

From the present data alone it is not certain whether the input virus dose is closely related with initial establishment of persistent infection. In earlier experiments (Nii and Kamahora, 1962) limited virus growth occurred in proportion to the inoculum dose and similar results were presented by Blackmore and Morgan (1967). Under the present experimental conditions, however, maximum virus growth occurred irrespective of the input virus dose. This discrepancy between earlier results and the present ones was caused by different experimental conditions. In previous experiments Earle's L cells were maintained in this laboratory and cultivated with medium consisting of 95 parts of Hanks' solution containing yeast extract and lactalbumin hydrolysate and 5 parts of bovine serum, while in the present

work cells obtained from Dr. Rose's tissue culture laboratory, Columbia University were cultivated with Eagle's Minimum Essential Medium supplemented with 10% calf serum. Although persistent infections were established under the latter conditions, they were not when the same cells were cultivated with 199 medium instead of Eagle's MEM. So far as Earle's L cells are concerned, these delicate culture conditions are related with the establishment of persistent infection of herpes simplex virus.

The persistent infections obtained in the present study were characterized by occasional appearance of increased virus synthesis and great cell destruction followed by regrowth of cells and also by a balance between virus and cells growth. This was a common feature during most of the period of infections, consisting of small atypical cycles of rise and fall in virus infectivity. No regular cycling patterns such as those shown by Hampar and Copeland with Chinese hamster cells (1965) were seen. The period needed for completion of one typical cycle in this study namely one to two months was much longer than that reported by the latter authors of ca. 16 days. One explanation of this difference may be the difference in the length of the cell doubling time which is very short for the Chinese hamster cells used for their experiments, being 16 to 14 hr.

Our cultures with persistent infection consisted of infected and noninfected cells. On histological examinations some but not all cells were found to have intranuclear inclusion bodies and in fluorescent antibody studies cells with and without viral antigen were observed. Moreover, virus free cells were occasionally found among the cells suspended in the fluid phase of cultures with persistent infection on subculturing them with medium containing antibody, although even by this method virus was often carried into the newly prepared cultures (in preparation). From these facts it is unlikely that a mechanism like lysogenicity of bacterial viruses operates to maintain the persistent infections in vitro.

Contamination with other kinds of agent has not yet been shown as a cause of persistent herpes infection. By electron microscopy only particles with the characteristics of herpes virus were observed and no other microorganism was observed.

It is noteworthy that in spite of the existence of considerable amount of infectious virus in the culture fluids, infection did not easily spread through the whole monolayer sheet but remained in an arrested state for a long period until a typical cycle of virus synthesis and great cell destruction appeared. In an earlier report (Nii and Kamahora; 1962) it was suggested that abortive infection occurred in some cells in a monolayer infected with a low virus input. This seems to be so in the present experiments also, as realized by the observation that in most cases infection started to spread out from a limited number of focal lesions and this occasionally led to widely disseminated infection through the whole monolayer. This means that a single virus infection of cells usually resulted in abortion while multiple infection of cells at the focal areas resulted in production of enough viruses to infect neighbouring cells. In this connection it is interesting that electron microscopy of sedimentable fractions of the fluid phase of cultures with persistent herpes infection showed very few particles with envelopes or particles of capsids with an electron dense core. However, there were many capsid particles with cores of low density which were labile outside the cells and presumably did not contain virus DNA (Nii et al., 1968a: Nii et al., 1968b).

It is not yet known why only a few cells escaped viral infection even at the most advanced stage of CPE. The supernatant of the culture fluids did not affect plaque formation of vaccinia virus and this coincides with the data of Blackmore and Morgan (1967). In the cellular fractions of cultures with persistent infection, however, these authors found a viral interfering factor resembling interferon in many of its properties. The role of interferon in maintaining persistent herpes infection in

the present study is ambiguous because at the advanced stage of CPE almost all the cells in the culture were killed. Therefore the few cells which were partially resistent to herpes simplex virus seemed to be enough to maintain this persistency. It is difficult to elucidate the cause of this partial resistence of a limited number of cells. This situation seems to be the same as that reported by Hampar and Copeland (1965). Contrary to the foregoing discussions Wadell and Siegel (1966) described a role of interferon, found in the supernatants of KB cell cultures with persistent herpes simplex infection, in maintaining viral persistency in the cultures. Further experiments to elucidate the role of interferon on herpetic persistency are now being planned.

As one possible mechanism of herpetic persistency in vitro Fernandetz (1960) suggested from his own experiment the appearance of the selected population of less susceptible cells causing a very slow virus spread in the culture. In the present study cells which had recovered from a primary infection were not resistent to a second infection and in the persistently infected cultures selection of cells resistent to infection with herpes simplex virus was not noticed during the past four years and even now accelerated virus synthesis and subsequent destruction of almost all cells in the cultures occurs. However, the possibility that either the host cell or the virus or both might undergo certain changes in the long period of more than four years after the establishment of the persistent infections cannot be excluded, while it is unlikely that alternate changes of the host cell and the virus occurred in each cycle of cell destruction and regrowth because such rapid changes of the two seem unlikely.

A gradual but definite changes in certain characteristics of herpes virus in cells with persistent infection was observed by Hinze and Walker (1961). Hampar (1966) also noted the appearance of a new variant in persistent ininfections. More recently a similar phenomenon has been encountered in the cultures with persistent infection in this study and aspects of host virus interaction with formation of a new variant will be discussed in a subsequent report.

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to Dr. H. M. Rose, Department of Microbiology, Columbia University, in whose tissue culture laboratory this work was started when the author was a postdoctoral fellow and also to Dr. S. Kato in this laboratory and Dr. C. Morgan, Columbia University for their encouragement. The work in Columbia University was supported by a grant from the Rockefeller Foundation.

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