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PERSISTENT INFECTION WITH HERPES SIMPLEX VIRUS IN VITRO. II. EFFECT OF ANTIBODY ON THE COURSE OF HERPETIC PERSISTENCY IN EARLE'S L CELLS[1,2]

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SUMMARY The effect of antibody on persistent infections of herpes simplex virus in Earle's L cells was investigated. (1) Addition of either human antiserum or human γ globulin to the medium of presistently infected cultures suppressed the spread of cytopathic changes and later the cultures appeared to be cured of infection. However, it was very hard to eliminate the virus from most cultures and the virus persisted for the various periods tested of 1 to nearly 8 months. Only a few cultures were cured of infection after antibody treatment for 7 to 8 months. (2) It was difficult to eradicate virus from cells even by successive subculture of persistently infected cells in medium containing antibody and the agent was pertinaciously associated with the cells in persistently infected cultures. When no antibody was present in successive subcultures, cytopathic changes generally appeared first in fresh cultures. This means that virus growth was greatly influenced by physiological conditions such as the age and cell population of the cultures. (3) A state resembling viral latency in man was also found in these subcultures. Namely, in some cultures without antibody infectious virus and cytopathic changes were only detected after a latent period of several months. (4) Comparative microepidemiological studies were made on herpes simplex virus in two cell strains, Earle's L and BSC1 cells. Persistent infection of BSC1 cells with herpes simplex virus was only established in the presence of antibody. The progressive virus spread in these persistent infections differed from that in Earle's L cells in the presence of antibody in the mode of cell to cell virus transmission. In the latter virus spread was very greatly arrested.

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

2 Most of this work was presented at the 15th Annual Meeting of the Society of Japanese Virologists in Chiba, on October 31, 1967.

INTRODUCTION

Presistent infections with herpes virus established under routine tissue culture conditions and maintained for a long period were first described in cell lines from the Chinese hamster by Hampar and Copeland (1965). Persistent infections with herpes simplex were also established in Earle's L cells without adding anti-herpes antibody to the culture medium and these infections were maintained for more than four years (Nii, 1969). In the latter infections a balance between viral and cell growth, in which atypical cycles of rise and fall in virus infectivity were shown, was the most common feature and occasionally increased virus synthesis and extreme cytopathic changes, followed by regrowth of the cells occurred in the cultures.

An in vitro system for persistent infections under culture conditions without antibody is very useful to analyse the close host cell-virus interrelationship of herpes simplex virus in vivo. However, this host-virus relation in vivo is maintained in a much more complexed *milieu* than that in vitro. Moreover a high level of circulating antibody against the agent is found in the blood of humans who harbor the virus. In herpetic infections in vitro, addition of antibody to culture media prevented spread of cytopathic changes by the virus more or less considerably and persistency was fairly readily established (Wheeler and Canby, 1959; Fernandez, 1960; Hinze and Walker, 1961; Szántó, 1963; Waddell and Sigel, 1966). The present investigation was on whether the persistency of herpes simplex virus in Earle's L cells was stable even in media containing antibody. The kind of cells involved in latent herpes infection in man are discussed.

MATERIALS AND METHODS

1. *Virus and infectivity titration*

To establish persistent infections of herpes simplex virus in Earle's L cells the -GCr Miyama strain was chosen (Nii, 1969).

2. *Cells*

Earle's L and BSC1 cell lines obtained from the tissue culture laboratory of Dr. H. M. Rose, Department of Microbiology, Columbia University were used. Earle's L cells were cultivated with Eagle's Minimum Essential Medium (MEM) or 199 Medium supplemented with 10% calf serum, while BSC1 cells were cultured in 199 Medium with 10% calf serum.

Many subcultures of persistently infected cells were prepared as described previously (Nii, 1969).

3. *Antibody and complement*

Human antisera with a neutralizing titer of $\times 128 - \times 256$ were used at a concentration of 5 or 10% in culture media. Human γ globulin (Cohn Fraction II) was purchased from Nutritional Biochemical Co. and 1 g of the poweder was dissolved in 20 ml of 199 Medium or MEM. The undissolved material was removed by three successive low speed centrifugations. Unless otherwise stated the supernatant was usually added to culture medium at a final concentration of approximately 5%.

Lyophilized guinea pig serum purchased from Nankai Pharmaceutical Institute was used as a source of complement.

RESULTS

1. *Effect of antibody on L cell cultures persistently infected with the -GCr Miyama strain of herpes simplex virus*

Since the first establishment of persistent infection of Earle's L cells with herpes simplex virus many subcultures were prepared, some of which were described in the previous report (Nii, 1969). Nine subcultures designated respectively as $L_{1'B}$, $L_{2''A}$, $L_{2''B}$, $L_{2'''}$, $L_{2''''}$, $L_{2'''''}$, $L_{4''A}$, $L_{4''B}$ and $L_{4'''}$ were used in this work. $L_{1'B}$ was a subculture made with cells suspended in the culture fluid of L_1. Similarly $L_{2''A}$ and $L_{2''B}$ were prepared using suspended cells of $L_{1'A}$ and $L_{1'B}$, $L_{2'''}$ with those of $L_{2''A}$ and $L_{2''''}$ with those of $L_{2'''}$ etc. Thus all five subcultures, $L_{2''A}$, $L_{2''B}$, $L_{2'''}$, $L_{2''''}$ and $L_{2'''''}$ were derived from L_2 while all three subcultures, $L_{4''A}$, $L_{4''B}$ and $L_{4'''}$ were descendants of L_4.

The normal growth medium of the above

TABLE 1. *Effect of antiserum on the persistence of herpes simplex virus in Earle's L cells*

Subcultures (named after the parental culture)	Period of culture in the presence of antiserum (Days)	Reappearance of CPE (Days after removal of antiserum)	Reappearance of infections virus (Days after removal of antiserum)
$L_{1'B}$	233	+ (70)	+ (61)
$L_{2''A}$	211	+ (55)	+ (45)
$L_{2''B}$	225	+ (38)	+ (38)
$L_{2'''}$	233	+ (61)	+ (38)
$L_{2''''}$	193	+ (53)	+ (46)
$L_{2'''''}$	30	+ (63)	+ (63)
$L_{4''A}$	233	−	−
$L_{4''B}$	225	−	−
$L_{4'''}$	225	−	−

nine cultures was replaced by medium containing antibody, i.e. MEM containing 5% calf serum and 5% human antiserum and the medium was renewed at 5 to 7 day intervals. From soon after addition of antiserum to the culture medium, the cytopathic changes in the persistently infected cultures gradually decreased and within one month the cytopathic foci could not be distinguished from overcrowded areas of non-infected cells. After growing the cultures in medium containing antibody for 30 to 233 days, cultures were grown in medium without antibody (Table 1). The culture fluid was occasionally titrated for infectivity and cells were examined microscopically every day to see whether herpes simplex virus had persisted in the cultures in the presence of antibody.

Infectious virus reappeared in the fluid phase of the six subcultures $L_{1'B}$, $L_{2''A}$, $L_{2''B}$, $L_{2'''}$, $L_{2''''}$ and $L_{2'''''}$ at the same time or a little before cytopathic changes appeared (Table 1). For example, in $L_{1'B}$ cytopathic changes became visible 9 days after detection of infectious virus in the culture fluid. Thus persistent infection reappeared in the six cultures when antibody was not present in the medium.

The appearances of infectious virus and the subsequent rises in titer of the fluid phases of the four cultures $L_{1'B}$, $L_{2''B}$, $L_{2'''}$ and $L_{2''''}$ are shown in Fig. 1. The pattern of the subsequent viral persistency in $L_{2''A}$ seen in

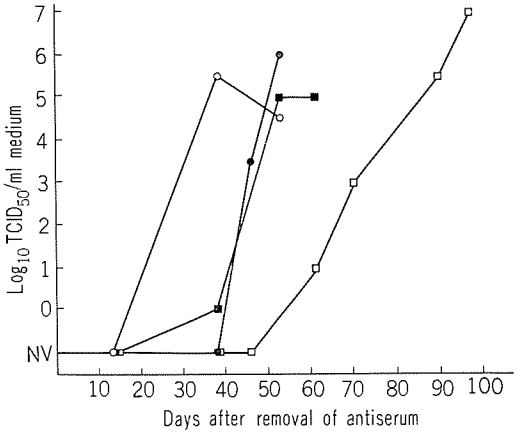


FIGURE 1. *Appearance of infectious virus in the fluid phase of four cultures after removal of antibody from the medium.*

Persistently herpes infected cultures, i.e. $L_{1'B}$, $L_{1''B}$, $L_{2'''}$ *and* $L_{2''''}$, *maintained in the absence of antibody were exposed to medium containing antibody for various periods as shown in Table 1. Then they were cultured without antibody and infectivity titrations were performed on the culture fluids.*

−□−···$L_{1'B}$, −○−···$L_{2''B}$,
−■−···$L_{2'''}$, −●−···$L_{2''''}$

Fig. 2 shows a maximum titer 76 days after removal of antiserum from the culture medium with subsequent atypical cycles of rise and fall in infective titer. On the contrary in all three cultures derived from L_4, i.e. $L_{4''A}$, $L_{4''B}$ and $L_{4'''}$, no infectious virus or cytopathic changes were detectable during more than six

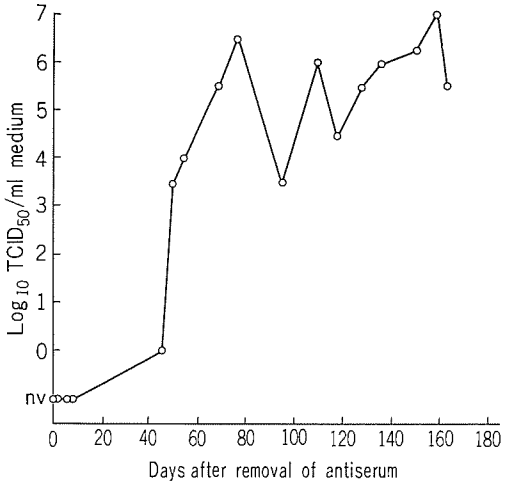


FIGURE 2. *Appearance and pattern of infectious virus in the fluid phase of culture $L_{2''A}$ after removal of antibody.*

months after removal of antiserum from the cultures.

The above data indicate that in $L_{1'B}$ and all cultures derived from L_2, herpes simplex virus persisted in the presence of antibody for periods of 1 to nearly 8 months while it was eradicated from all three cultures derived from L_4.

2. *Persistence of herpetic infection in two series of persistently infected L cell cultures*

In the former section it was shown that in several cultures derived from culture L_2 herpes simplex virus persisted in medium containing antibody. Culture L_2 was made on November 25, 1964 (Nii, 1969) and several successive subcultures were prepared from it.

Two experiments were made to obtain virus free cells from persistently infected cultures by antibody treatment. Both were unsuccessful but an interesting finding was made in these cultures on the appearances of infectious virus in the fluid phase and cytopathic changes after removal of the antiserum.

A diagram of one experiment is shown in Fig. 3. Culture C_1 was at the fifth generation of subculture from L_2. It was prepared by low speed centrifugation of the fluid phase of a subculture of L_2 at the fourth generation, resuspension of the cells (3×10^5 cells) in fresh medium containing 5% human antiserum and inoculation of the suspension into a 50 ml square bottle. Most cells were distributed separately on the wall of the vessel for at least three days, as revealed by microscopic observation. This separate distribution of the cells was achieved by limiting the cell number in the inoculum and was aimed to prevent viral transmission from infected cells to normal ones since the medium contained antibody. Moreover, it seemed likely that under these conditions infected cells would die and eventually infectivity associated with cells would be lost so that a virus-free culture would be obtained. As indicated by the thick line in Fig. 3 the cells of culture C_1 were maintained in medium containing antibody for 8 days. Then after 5 days further cultivation of C_1 without antibody a new culture C_2 was made in the same way as in the preparation of C_1. After 11 days cultivation of C_2 with antibody a further subculture was made, named C_3. After cultivation of C_3 with antibody for 7 days and then without antibody for 4 days, a fourth culture, C_4, was made. Thus four clutres, C_1, C_2, C_3 and C_4 were prepared successively.

Contrary to expectation, virus was not eradicated by successive subculture with antibody, and severe cytopathic changes appeared in C_4 and moderate changes in C_3. These changes were observed after 13 days culture of cells in antibody free medium, as shown in parenthesis in Fig. 3. The culture fluids removed at this time (i.e. March 21, 1967) from C_3 and C_4 had titers of 10^7 /ml and $10^{7.5}$/ml, respectively.

However, no cytopathic changes were detected at this time in C_1 and C_2. Both cultures should contain the infectious agent since the cells of C_3 and C_4 which became severely infected originated from culture C_1 through C_2. The absence of cytopathic changes in C_1 and C_2 was not due to poor culture conditions and cells in C_2 grew vigorously, many becoming

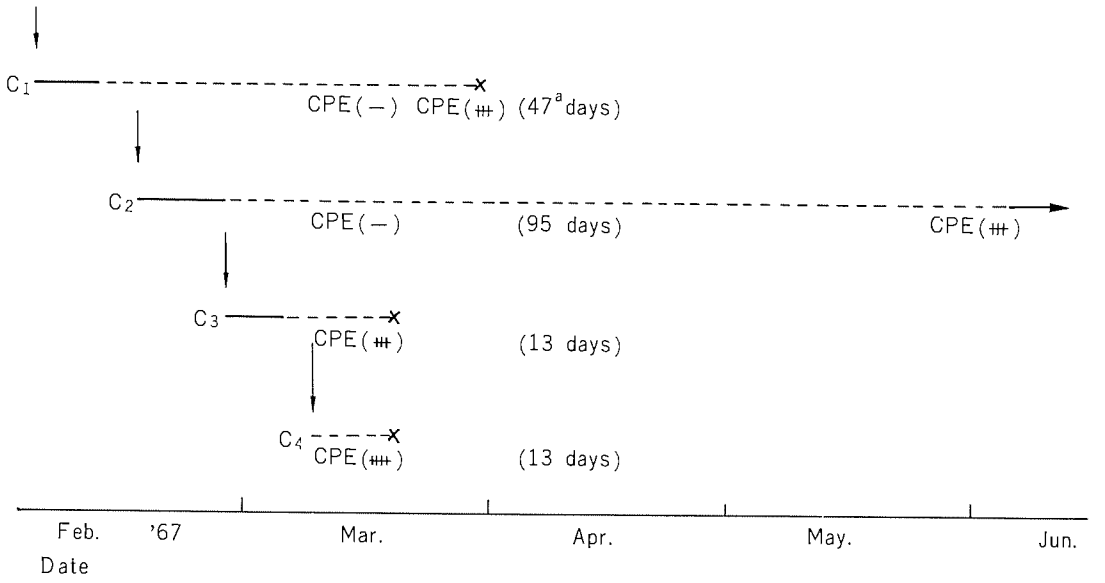


FIGURE 3. *Preparation of four successive subcultures and appearance of cytopathic changes in them after removal of antibody.*
——···*antibody treatment*, — ~ +++ ···*extent of CPE*,
a···*days from removal of antibody to appearance of CPE*,
×···*cultivation stopped*

suspended in the fluid phase due to overgrowth.

To see whether these floating cells harbored the infectious agent, the culture fluid (6 ml, containing 1.6×10^6 cells) was removed. A 2 ml aliquot of the fluid were freeze-thawed five times and the resulting sample was titrated. Some of the cells, (6.5×10^5) in another aliquot of the fluid were harvested by centrifugation and suspended in 20 ml of fresh MEM solution containing 5% calf serum. Then 2 ml aliquots of the suspension were inoculated onto FL cell monolayers in test tubes. No infectious virus was detected by either method. The viral infectivity of cells attached to the glass was not examined but it should be mentioned that the viral growth in C_2 was extremely limited and the amount of infectious agent present was very small. The appearance of cytopathic changes in C_2 was greatly delayed, only becoming visible 95 days after removal of antiserum.

Results were similar in the second experiment, which is shown schematically in Fig. 4. Culture D_1 was prepared on December 26, 1966 and was a third generation subculture from L_2 cultured continuously in medium containing antibody. After one month antibody was removed and after further cultivation for 8 days a new subculture, D_2 was prepared from the cells suspended in the fluid phase of D_1. D_2 was cultured in fresh medium with antibody. After 8 days cultivation of D_2 with antibody it was cultured without antibody.

As in the first experiment cytopathic changes appeared first in the younger culture, D_2, being observed 109 days after removal of antibody. At this time the culture fluid had a titer of $10^{5.5}$ $TCID_{50}$/ml (Fig. 4). However, no cytopathic changes or infectious virus were detected in D_1 at this time. Accordingly attempts were made to activate the virus in D_1 by changing the medium every day for one week and then in the routine way by changing it every few days. Cytopathic changes started

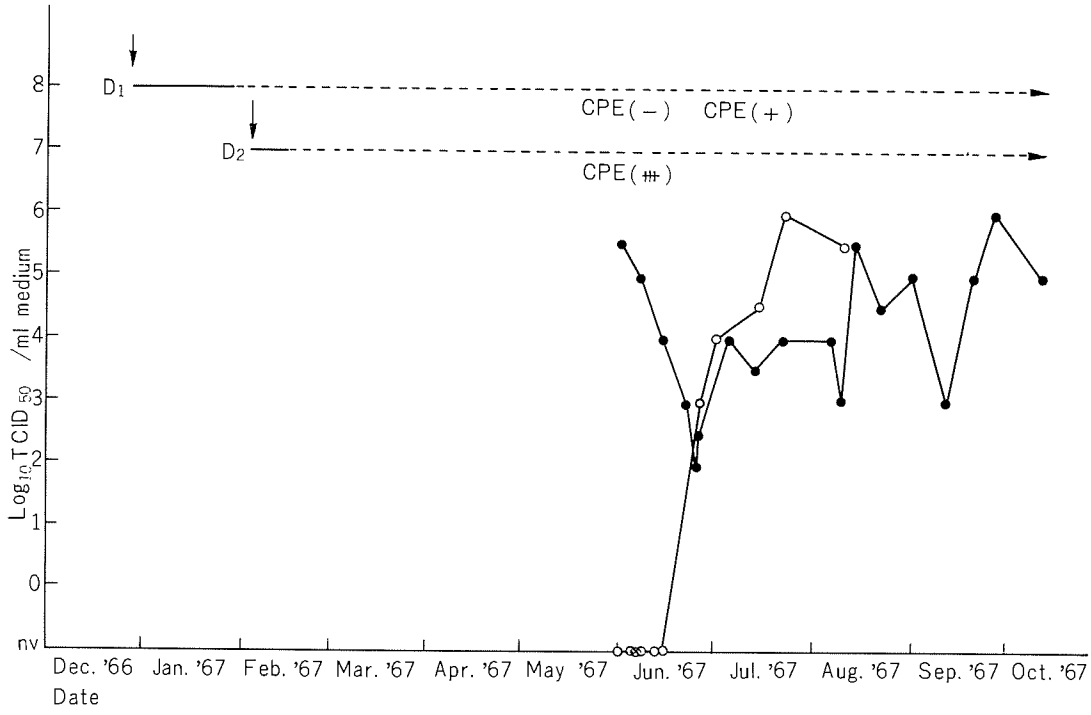


FIGURE 4. *Preparation of two successive subcultures and development of cytopathic effects and infectivity in them.*
——···*antibody treatment*, —~卌···*extent of CPE*,
—○—···*infective titer in the fluid phase of* D_1,
—●—···*infective titer in the fluid phase of* D_2

to appear after one month, that is 156 days after removal of antibody. The infectivity titers of culture fluids of D_1 and D_2 for several months subsequently are shown in Fig. 4.

The following conclusions are drawn from these two experiments. (1) It is difficult to eradicate virus from cells even by successive subculture of persistently infected cells in medium containing antibody and the agent is pertinaciously associated with the cells in persistently infected cultures. (2) On successive subculture of persistently infected cells in medium without antibody cytopathic changes generally appear first in fresh cultures. This means that virus growth in the cultures is greatly influenced by the physiological conditions of the cells. A similar finding was reported previously (Nii, 1969). (3) Occasionally cells grew well while virus growth was arrested as in culture C_2 in the first experiment. This means that under some conditions there is a disparity between cellular proliferation and viral growth (Taniguchi, 1966).

3. *Effect of concentrated antibody on herpetic persistency in Earle's L cells*

An experiment was made to see whether persistent infections, such as those shown in subcultures of L_2 could be maintained at much higher antibody levels. Human γ globulin (NBC) was added to MEM supplemented with 5% calf serum to give a neutralization titer of the culture medium against herpes simplex virus of $\times 64 - \times 128$. This antibody level is approximately the same, or slightly less than that normally found in adult human subjects.

Lyophilized guinea pig serum resolved in MEM was also added to the medium at a concentration of 8 hemolytic units of complement per 0.2 ml (Yoshino and Taniguchi, 1964).

One culture originally derived from L_2 was maintained in this medium for 33 days. Culture fluid was removed for titration during this period and also for 20 days before and after this period. Results are shown in Fig. 5, the period of culture in the special medium being indicated by a thick line.

Results show that herpes simplex virus could not be eradicated from the culture but persisted for at least one month in the presence of approximately the same level of antibody as that persent in man.

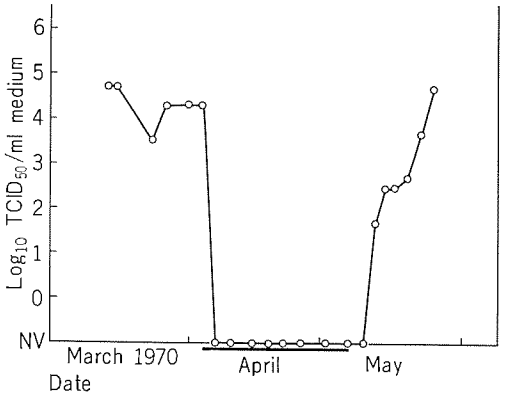


FIGURE 5. *Effect of a high level of antibody on herpetic persistency in Earle's L cells.*
——··· *period of antibody treatment*

4. *Ease of establishment of persistent infections of herpes simplex virus in Earle's L cells using antibody*

Infection of Earle's L cells with the Miyama strain of herpes simplex virus did not always result in persistent infections. Achievement of persistent infection seemed to depend largely on the kind of culture medium. Persistent infections were never obtained with 199 Medium while with Eagle's MEM they could be established (Nii, 1969). However, using antibody persistent infections were easily established even in Earle's L cells in 199 Medium.

Four L cell monolayers, No. 1, 2, 3 and 4, were prepared in 30 ml Falcon plastic bottles. The virus used was derived from a persistently infected L cell culture previously designated as $L_{4'}$ (Nii, 1969). The culture fluid was removed and 1 ml of the supernatant (4.6×10^4 $TCID_{50}$) was inoculated into each of the four cultures with 3 ml of fresh 199 Medium. The next day the medium was replaced by growth medium (199 Medium supplemented with 10% calf serum). Cytopathic changes became visible 2 days later and were evident in all the cultures 3 days later. Five days after virus inoculation when cytopathic changes were marked, culture of No. 1 and No. 2 in medium containing antibody (199 Medium supplemented with 10% human serum) was started. No. 3 and No. 4 were cultivated further in antibody-free medium (Fig. 6).

Persistence of virus in No. 1 and No. 2 was examined one month later by removal of antibody. Five days after removal of antibody No. 1 was divided into 4 subcultures, No. 1a, 1b, 1c and 1d. Cytopathic changes appeared in all these cultures (1a-d and 2). Infectivity titrations were performed with the culture fluids of No. 1a, 1b, 1c and No. 2. Results are shown in Fig. 7. Then No. 2 was again cultured with antibody.

Virus persistency in No. 2 was reexamined after 66 days culture with antibody. No. 2 was divided into portions No. 2a, 2b and 2c and antibody was removed from 2c. Clear cytopathic changes soon appeared in 2c.

In two successive cultures of No. 2 and 2a virus persisted for 211 days, in the presence of antibody. The infectivity curve of the culture fluid of No. 2a after removal of antibody is shown in Fig. 8.

These results showed that persistent infections of herpes simplex virus in Earle's L cells were readily established in 199 Medium containing antibody. However, persistent infections were not established without antibody. Cultures No. 3 and No. 4 were not treated with

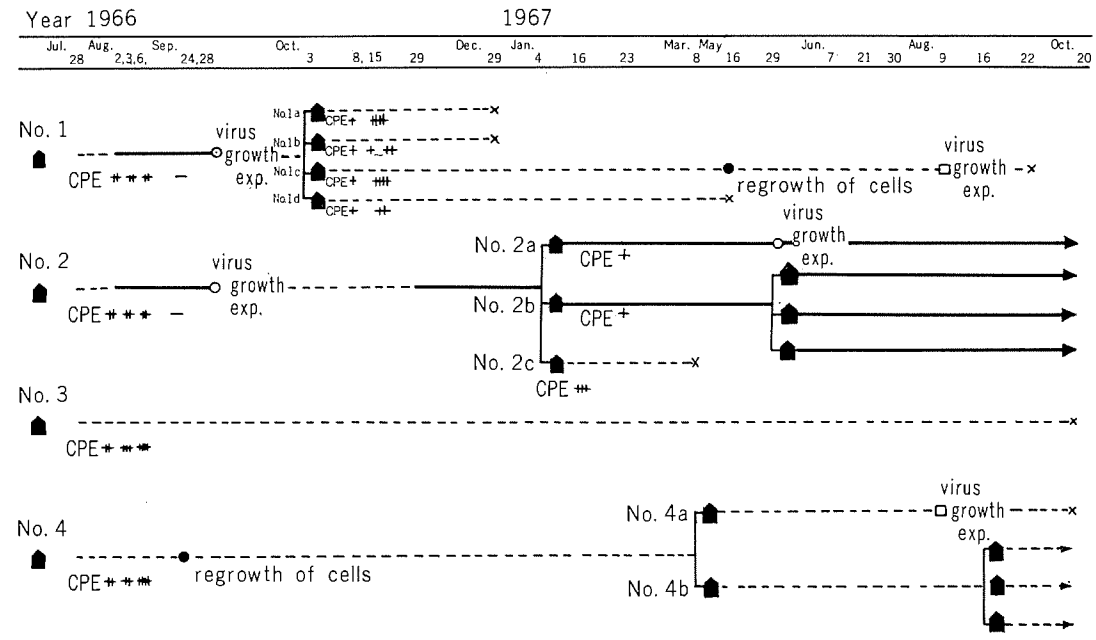
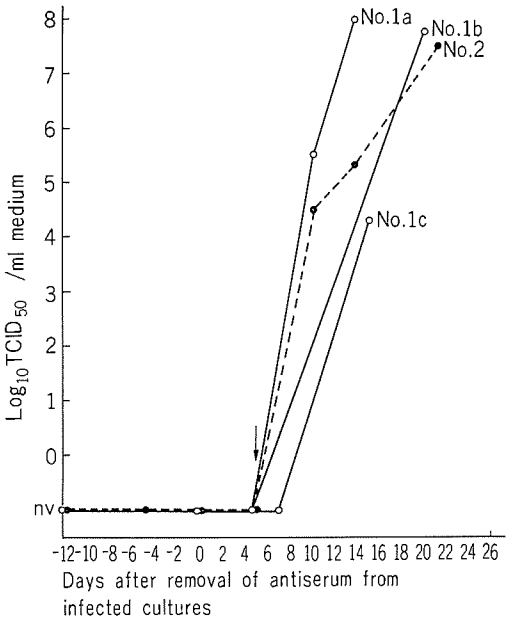


FIGURE 6. *Persistent infection of herpes simplex virus in Earle's L cells in 199 Medium with antibody* ——···*antibody treatment,* —~卌···*extent of CPE,* ●···*regrowth of cells detected,* ○···*exteriments made to test for persistency in the cultures,* □···*experiments made to test susceptibility of cured cells*



antibody, and the virus was lost soon after the appearance of maximal cytopathic changes following to the first virus inoculation. Further, the persistent infections established by antibody treatment were not maintained after removal of the antibody. This was shown with cultures of No. 1a, 1b, 1c, 1d and No. 2c.

In some infected cultures in antibody-free medium a few cells survived and formed colonies without any sign of virus infection and later the whole wall of the bottom of the culture bottle became covered with cells. Attempts

◀

FIGURE 7. *Appearance of infectious virus in the fluid phase of cultures No. 1; 1a, 1b, 1c and 2 after removal of antibody.*

—○—···*growth in No. 1 and its subcultures (No. 1a, 1b and 1c)*

—●—···*growth in No. 2*

The arrow indicates the date when No. 1 was subcultured.

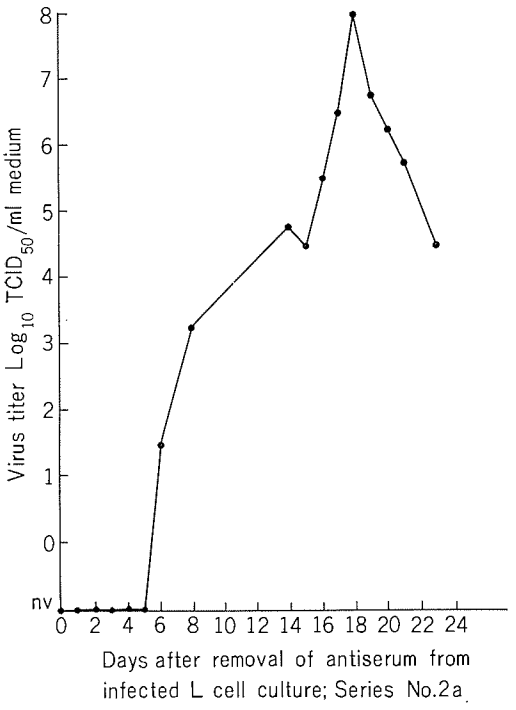


FIGURE 8. *Appearance of infectious virus in the fluid phase of culture No. 2a after removal of antibody.*

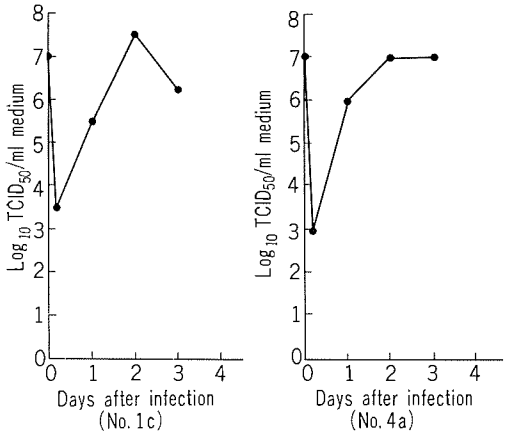


FIGURE 9. *Reinfection with cured cells in cultures No. 1c and 4a.*

Two ml of viral sample (titer, 10^7 $TCID_{50}$) were inoculated into each culture. After adsorption for 2 hr the cells were washed three times with 199 Medium without serum and then 6 ml of 199 Medium supplemented with 5% calf serum was introduced onto each culture. Infectivity was measured 4 hr and 1, 2, 3 and 4 days later.

to detect infectious virus in these cultures were unsuccessful. This phenomenon was observed with cultures No. 1c and No. 4. However, these cured cells were not resistant to reinfection with herpes simplex virus. Thus cultures No. 1c and No. 4a were inoculated with 2 ml of virus material derived from No. 2a (titer, 10^7 $TCID_{50}$). The virus in the culture fluids was titrated and results are shown in Fig. 9.

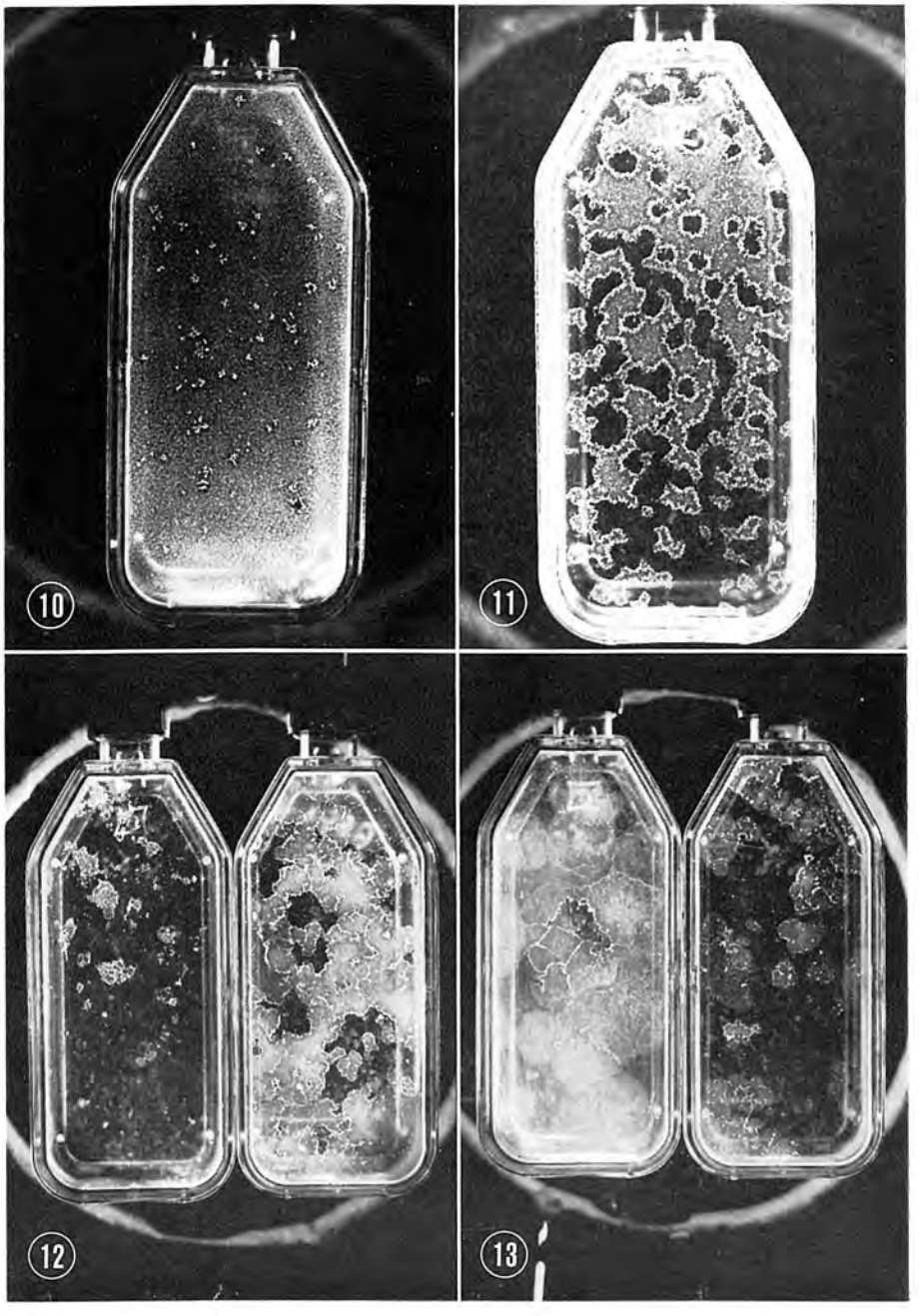
No further observations were made on these cultures. However results show that antibody is essential for establshing persistent infections of herpes simplex virus in Earle's L cells in 199 Medium.

5. *Establishment of persistent infections of herpes simplex virus in BSC1 cells in medium with antibody*

BSC1 cells originally derived from an African green monkey and Earle's L cells derived from mouse connective tissue have different morphological appearances in vitro. The former are epithelial while the latter are fibroblastic. The following experiment was performed to examine the host cell-virus interaction between BSC1 cells and the —GCr Miyama strain of herpes simplex virus and the differences in the spread of this agent in BSC1 cells and Earle's L cells.

The above experiments with Earle's L cells show that virus growth is generally less in older cultures. Accordingly, in an attempt to establish persistent infections without using antibody aged BSC1 cultures were used. Four BSC1 monolayers were prepared in 30 ml Falcon plastic bottles. Two months later 1 ml of virus sample derived from L_2 (titer, $10^{5.5}$ $TCID_{50}$) was inoculated into each culture. After adsorption for 1 hr the inoculum was removed. Normal growth medium, i.e. 199 Medium containing 10% calf serum, was introduced into two cultures while the other two were cultured with the same medium supplemented with approximately 0.25% human γ globulin (NBC).

Plaques were detected microscopically 2 days later and became visible to the naked eye 5 days


10
11
12
13

later. The extent of cytopathic changes differed in the cultures with and without antibody.

In the latter wide-spread infection occurred and after one week almost all the cells had degenerated. Regrowth of cells did not occur in either culture.

However in cultures with antibody formation of secondary plaques was more or less suppressed and the primary plaques produced by the first inoculum of virus gradually increased in size, showing a dendritic appearances (Fig. 10). Soon big plaques were observed as vacant areas due to detachment of degenerated cells. The plaques increased in size and fused together (Fig. 11) and eventually most of the cells became infected and were detached from the wall of the bottle. Meanwhile a few cells which escaped infection proliferated and soon colonies of non-infected cells were formed. Finally most of the bottle was again covered with a monolayer sheet. During this cellular regrowth the proliferating cells came in contact with infected cells and this led to a new cycle of infection in the culture.

◀

FIGURES 10–13. *Presistent infection of BSC1 cells with herpes simplex virus in the presence of antibody.*

Four, two month old BSC1 monolayers in 30 ml Falcon plastic bottles were infected with 1 ml of a virus sample derived from L_2 *(titer,* $10^{5.5}$ *TCID*$_{50}$*). After adsorption for 1 hr two cultures were incubated with 6 ml of fresh 199 Medium supplemented with 10% calf serum while the other two were incubated with the same medium containing antibody. Persistent infections were established in the latter cultures.*

Fig. 10 shows a 30 ml Falcon plastic bottle photographed two weeks after virus inoculation. About one hundred plaques are visible. Fig 11 shows the appearance of a BSC1 monolayer one month after infection. Most plaques have fused. Three months after virus inoculation most cells are detached from bottles, as shown in the left bottle in Fig. 12. The monolayer in the right bottle had a patchy appearance since spread of cytopathic changes was slower than in that on the left. Regrowth of cells soon started in the bottle on the left and 39 days after the picture in Fig 12 an almost complete monolayer was formed, as shown by the left bottle of Fig. 13. In the right bottle in Fig. 12 cytopathic changes progressed further and fewer cells were found, as shown in the bottle on the right in Fig. 13.

This mode of persistent infection in medium containing antibody, consisting of progressive spread of cytopathic changes with subsequent regrowth of noninfected cells, was observed in both the cultures. However, one remarkable difference was noticed between them. In the left bottle in Fig. 12, cytopathic changes progressed more rapidly and consequently regrowth of cells started earlier than in the other bottle (Fig. 13). This fast spread of virus infection in one bottle was found to be caused by the appearance of the +GC variant (Nii and Kamahora, 1961) by mutation in this culture. Similar changes in the severity of herpetic infections in the presence of antibody caused by the appearance of a new variant were also found by Hampar (1966) with the MAL line of Chinese hamster cells.

In the above experiments persistent infections were established in BSC1 cell monolayers in medium with antibody but not without antibody. The progressive virus spread in these persistent infections differed from that in Earle's L cells in the presence of antibody in the mode of cell to cell virus transmission, i.e. in the latter virus spread was very greatly arrested.

DISCUSSION

In the present investigations on persistent infections of herpes simplex virus various experimental conditions were tested for growth of the virus (different kinds of host cells and basal media with and without antibody). These are summarized in Table 2. The —GCr Miyama strain of herpes simplex virus was used throughout at the low virus input of 10^4–10^6 $TCID_{50}$ per bottle for the first infection of cells.

As shown by Table 2 persistent infections were always established by addition of antibody. Without antibody, however, persistent infection was only observed in Earle's L cells in MEM containing 10% calf serum. The establishment and some characteristics of these presistent infections were presented in detail

TABLE 2. *Experimental conditions tested to establish persistent infection of herpes simplex virus in vitro*

Host cell	Medium	Antibody	Establishment of persistent infection
Earle's L	MEM	+	+
		−	+ or −
	199	+	+
		−	−
BSC1	199	+	+
		−	−

in the previous report (Nii, 1969).

Herpes simplex virus persists in humans who have a sufficiently high level of circulating antibody. Therefore attempts were made to see whether persistent infections established in vitro in the absence of antibody could be maintained in medium containing antobody. Addition of either human antiserum or human γ globulin to the medium of persistently infected cultures caused reduction in the cytopathic changes and later the cultures appeared to be cured of virus infection. However, it was usually found very difficult to eliminate the virus from cultures by antibody treatment. For example, one culture was treated with antibody for 233 days, but when the cluture was then grown in medium without antibody cytopathic changes reappeared in the cell sheet after about 2 months and virus was recovered from the culture fluid. However, virus was eradicated from a few cultures after antibody treatment for a similar long period. Thus a stable host cell-virus interaction was maintained in the former cultures but not in the latter. Various host cell-virus relations are probably produced in persistently infected cultures nourished in medium without antibody by selection of viruses and/or cells, during the cycle of destruction of the cells by the virus and subsequent regrowth of the cells. Successful maintenance of virus persistency for a long period in medium containing antibody may depend on the stability of the relations between host cells and virus.

A state resembling that of viral latency of herpes simplex virus in man was often encountered. Cells suspended in the fluid phase of persistently infected cultures were harvested, resuspended in fresh medium containing antibody and introduced into new bottles. In this way several subcultures were prepared in medium containing antibody and these were then grown without antibody. This process seemed to establish cultures free of virus. However, after a latent period cytopathic changes reappeared and the virus recovered. In one typical case the viral latency period was five months. Daily renewal of the medium seemed to accelerate the reappearance of CPE in one culture and this suggests that improvement in the physiological conditions of the cells results in reactivation of viral growth. This latent herpetic infection in vitro seems to resemble the lysogenic state of tempelate bacteriophages although lysogenicity may be not responsible for this herpetic latency in vitro. The mechanism of this phenomenon requires elucidation.

Two other interesting phenomena were found with persistently infected Earle's L cells subcultured with antibody and then grown without antibody. First, virus growth was generally more active in freshly prepared cultures than in older cultures. Probably virus growth is more active in fresh subcultures even in medium containing antibody and consequently on removal of antibody cytopathic changes appear much earlier in fresh cultures. Thus virus growth in persistently infected cultures depends greatly on some physiological conditions of the cells caused by the age of the cultures or their cell populations, or both. Second, cells occasionally grew vigorously without simultaneous appearance of cytopathic changes, indicating a disparity between cell proliferation and virus growth.

These in vitro phenomena may help to explain the latency and recurrence of herpes simplex virus in vivo. Various direct or

indirect stimuli of skin tissue (e.g. ultraviolet light, pyrexia, hormone treatment or neurosurgery) may cause physiological changes of the cells harboring the virus and initiate virus growth. Moreover, some disparity between normal physiological activities of the cells and virus growth undoubtedly exist in tissues in vivo. In healthy subjects herpes simplex virus is never recovered from local areas in periods between recurrences of disease indicating that virus growth is strongly arrested in some manner although epithelial cells are proliferating in the basal layer of the epidermis and other cells in the cutaneous and extracutaneous tissues are functioning well.

Comparative studies were made on infection of two cell lines, Earle's L and BSC1 cells, with herpes simplex virus. These were to see what kinds of cells serve as host cells for herpetic latency in man in vivo. In the BSC1-Miyama strain system, persistent infections were only established when antibody was present in the culture medium and were characterized by gradual but progressive development of cytopathic changes in monolayer sheets. Viral spread in persistently infected Earle's L cells was very different in that cytopathic changes were suppressed soon after the beginning of antibody treatment and could hardly be detected microscopically.

Persistent infections like that in the BSC1 cell line in the presence of antibody have also been reported with HeLa and other cell lines (Wheeler and Canby, 1959; Hinze and Walker, 1961; Szántó, 1963). This type of persistent infection, however, is not a good model of herpetic infection in human for the following reasons. First, infectious virus is readily demonstrated in persistently infected cultures while it is not usually possible to isolate the agent from human skin tissues during a period between recurrences. Second, if this type of persistent infection occurred in vivo it would always be accompanied by chronic focal inflammation. Therefore persistent infection of Earle's L cells seems to be a better model of that in man. Thus it seems likely that Schwann or satellite cells, rather than nerve cells or epithelial cells in the skin, are host cells during the latent period. The virus might be harbored by physiologically inactive cells such as Schwann or satellite cells for a long period in man. Then when virus growth is initiated in these cells under appropriate conditions caused by various stimuli and infections, viruses would be released, and neighbouring epithelial cells would be infected. Vigorous virus production accompanied by cell destruction would then occur in the epithelial cells.

Persistent infection of Earle's L cells with various viruses have been reported; WEE (Chambers, 1957), Newcastle disease (Henle et al., 1958), mumps (Henle et al., 1958), vaccinia (Randall and Ryden, 1960) and polyoma (Balducci and Morgan, 1960; Henle et al., 1963). All these persistent infections were established without antibody. The persistent infections with three DNA viruses of vaccinia, polyoma and herpes simplex were characterized by more or less extensive cycles of cellular destruction followed by regrowth of cells, except one report by Balducci and Morgan (1960) in which continuous production of polyoma virus with no observable CPE was shown. Cultures of cells persistently infected with polyoma virus were cured after months of continuous treatment with antipolyma serum (Henle et al., 1963), while it is very hard to eliminate herpes simplex virus by antibody treatment, as shown in present investigation. With vaccinia virus Randall and Ryden (1960) observed a prolonged non-infectious period before cell degeneration due to adapted viruses. This phenomenon closely resembles the herpetic latency observed here in vitro. However, one difference between the persistent infections with vaccinia and herpes simplex is that the —GCr strain of herpes simplex virus could infect L cells from the first virus inoculation and serial virus passage was easy in this cell line (Nii, 1969). At present it is uncertain whether the persistent infections with the three DNA viruses are established by the same mechanism.

The problems of what regulates herpetic latency in man, what precipitates recurrent eruptions, what kind of cells harbor the virus and whether these are Schwann or satellite cells have been discussed in various reviews (Roizman, 1964; Paine, 1964; Lwoff, 1966). Various factors, such as the level of antibody, interferon, variation of the virus and the physiological conditions of the host cells seem to be involved. The present work shows that the kind of cells and their physiological conditions are very important in latent herpes infections.

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