Persistent Cyclic Herpes Simplex Virus Infection In Vitro

II. Localization of Virus, Degree of Cell Destruction, and Mechanisms of Virus Transmission

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Received for publication 29 December 1965

ABSTRACT

HAMPAR, BERGE (National Institute of Dental Research, Bethesda, Md.). Persistent cyclic herpes simplex virus infection in vitro. II. Localization of virus, degree of cell destruction, and mechanisms of virus transmission. J. Bacteriol. 91:1959–1964. 1966. The localization of virus, degree of cell destruction, and mechanisms of virus transmission in persistent herpes simplex virus-infected cultures were studied. The major fraction of infectious virus was associated with the medium and a minor fraction was associated with the attached cells. Virus in the medium was further separable into a sedimentable (cellular) fraction and a nonsedimentable (extracellular) fraction. The sedimentable fraction was comprised of cellular debris, most of which appeared to contain viral antigen, and intact cells of which less than 10% contained infectious virus. Cell destruction during the cycle involved more than 99.9% of the maximal number of cells present. Infection could be transmitted by extracellular virus, cell-to-cell transfer, and reattachment of infectious cellular material. The results indicated that transmission by reattachment was probably mediated through the cellular debris rather than the intact cells.

The establishment and general characteristics of Chinese hamster cell cultures (MAL cell line) persistently infected with herpes simplex virus (HSV) have been described (5). Briefly, these cultures, which have thus far been maintained more than 18 months, display cycles of virus synthesis with accompanying cell destruction followed by regrowth of the cells. The infected cultures do not require the presence of antibody in the medium for maintenance, and can be passaged by use of normally employed techniques. This report further characterizes these persistent infections, and attempts to localize the infectious virus and estimate quantitatively the amount of cell killing and the mechanisms by which the virus is transmitted.

MATERIALS AND METHODS

Cell cultures. The persistently infected MAL cells, tissue culture techniques, including observations of the degree of virus induced cytopathic effects (CPE), and the media employed were described previously (5). Cultures were also maintained on media supplemented with either 20% calf serum or 20% fetal bovine serum.

Virus titrations. Virus was titered by the tube dilu-

tion method ($TCID_{50}$) in HeLa cells (5), and as plaque-forming units (PFU) in MAL cells. For titration in MAL cells, monolayer cultures were inoculated with 1 ml of virus diluted in Eagle's medium containing 10% inactivated calf serum (ECS), and incubated for 2 hr at 37 C on a horizontal shaker moving at slow speed. The viral inoculum was removed and replaced with 4 ml of ECS containing 2% rabbit anti-HSV serum. The cells were fixed for 5 min with methanol, stained with Giemsa stain, and the plaque numbers were recorded at 4 days by which time plaques 2 to 3 mm in diameter were present.

Degree of cell destruction and virus synthesis. Approximately 5×10^5 trypsin-dispersed cells from persistently infected cultures were seeded in 60-mm plastic petri dishes with 4 ml of medium. These cultures were refed every 2 days. At 1- to 2-day intervals, the medium was collected from each of two plates, pooled, and a portion was removed for virus titration. The remainder of the medium was centrifuged at 800 × g for 10 min. The sediment which contained intact cells and large fragments of disintegrated cells will be collectively referred to as the cellular fraction, and the supernatant which contained free virus or virus associated with small cell fragments, or both, will be referred to as the extracellular fraction. The terms cellular and extracellular are used merely to delineate the two fractions

obtained after centrifugation under these conditions. The cellular fraction was resuspended in 2 ml of 0.01 M phosphate buffer containing 0.85% NaCl and 1% heat-inactivated calf serum at pH 7.2 (PO₄ buffer), and was disrupted by sonic oscillation (Sonifier type LS 75, Branson Instruments, Inc., Stamford, Conn.) for 10 sec. The virus titers in the extracellular and disrupted cellular fractions were determined. The cells remaining attached to the petri dish were removed with 0.25% trypsin, the plates were washed two times with Hanks' balanced salt solution (BSS), and the suspensions were pooled and centrifuged at 800 $\times$ g for 10 min. The cells were resuspended to 2 ml in growth medium, and 0.5 ml was removed for counting in a Coulter counter. The cell counts varied to within 1 to 3% of similar counts by hemocytometer. Rabbit anti-HSV serum was added to the cell suspension at a final concentration of 10%, followed by incubation for 30 min at 37 C on a horizontal shaker. The bulk of residual antibody was removed by three cycles of resuspension and centrifugation. The virus titer in the final wash was 1% or less of that associated with the cells. The sediment from the final wash was resuspended in 2 ml of PO₄ buffer, and 0.5 ml was removed for counting. The remainder of the cells were disrupted by sonic treatment, and the virus was titered. Virus was titered in HeLa cells and calculated to represent $TCID_{50}$ of virus per plate.

Susceptibility of virus in the medium to neutralization by antibody. The susceptibility to neutralization by antibody of infectious virus in the supernatant (extracellular) and sedimentable (cellular) fractions of the medium was studied by titration in HeLa and MAL cells. Portions of the extracellular fraction were treated at 37 C for 1 hr with either 10% anti-HSV rabbit serum (antibody) or normal rabbit serum. The cellular fraction was washed two times by resuspension and centrifugation in medium, and the final wash was titered. The sediment was resuspended in the final wash, and the virus was titered. A portion of the cell suspension was centrifuged, resuspended in PO₄ buffer, and disrupted by sonic oscillation. Examination under phase contrast indicated that over 90% of the cells were disrupted by this procedure. Portions of the nondisrupted and disrupted cellular fractions were treated at 37 C with either anti-HSV serum or normal rabbit serum at a final concentration of 10% and were titered for virus. The nondisrupted anti-HSV serum-treated fraction was centrifuged, and the virus titers in the supernatant and sedimentable fractions were determined separately. The virus titers were corrected to represent 1 ml of the original medium.

Reattachment of infectious cellular material. Millipore filter chambers were constructed by cementing type HA (0.45-μ pore diameter) filters to one side of a nontoxic Plexiglas ring, followed by sterilization under ultraviolet light. One chamber, with the filter side down, was placed in the center of a monolayer culture of MAL cells. The chambers and parallel cultures without chambers were inoculated with 0.1-ml dilutions of various virus-containing fractions, and 2.5 ml of medium was added to each culture. The chambers remained firmly attached to the cell sheets so that the distance between the inoculum within chambers and the underlying cells was the thickness of the filter (150 μ ± 10 μ). The 2.5 ml of medium employed resulted in a level which avoided fluid exchange between the top of the chamber and the surrounding medium. Cultures were refed three times per week, and were maintained for 2 weeks to determine the virus titers in the presence and absence of chambers.

Fluorescent-antibody studies. The indirect technique as described by Hampton (6) was used. The rabbit anti-HSV sera and human globulin employed were previously described (5). Fluorescein-labeled and unlabeled goat anti-rabbit and anti-human globulins were obtained commercially (Microbiological Associates, Inc., Bethesda, Md.). All sera and globulins were absorbed at 37 C for 1 hr with disrupted uninfected MAL cells prior to use. Slides were examined under a Zeiss Opton microscope equipped with a mercury lamp (Osram OB 0200) and exciter filters UG1 and BG12.

Uninfected MAL cells were employed as control cells. Controls consisted of using preimmunization rabbit sera in place of immune sera, blocking with unlabeled immune globulins, attempts to stain cells treated with immune rabbit sera with fluorescein-labeled anti-human globulin and the converse, and attempts to stain cells directly with fluorescein-labeled globulins. All controls were negative, indicating the specificity of observed fluorescence for HSV antigen.

RESULTS

Degree of cell destruction and relationship of cell proliferation to virus synthesis. Figure 1 shows the results of experiments designed to study the relationship of cell proliferation and virus synthesis, and to quantitatively estimate the amount of cell destruction during the cycles of virus synthesis. To insure that the cultures used at each time interval studied were parallel with respect to their cycling patterns, two cultures were maintained throughout the experimental period, and their virus titers in the medium and their CPE were continually monitored (Fig. 1A). The CPE, as previously described (5), refers to the degree of cell destruction evident in the remaining attached cells irrespective of the number of cells present. Figure 1B shows the virus titers in the cellular and extracellular (Super.) fractions of the medium, and in the remaining attached cells. Figure 1C shows the number of attached cells at each time interval studied, and the ratio of the attached cell number to infective virus in these cells. When the titers in the medium from the two cultures maintained throughout the experimental period (Fig. 1A) are compared with similar titers from the cultures used at each time interval (Fig. 1B), it is evident that the cultures employed were parallel with respect to their cycling patterns. From Fig. 1B and 1C, certain general conclusions could be made
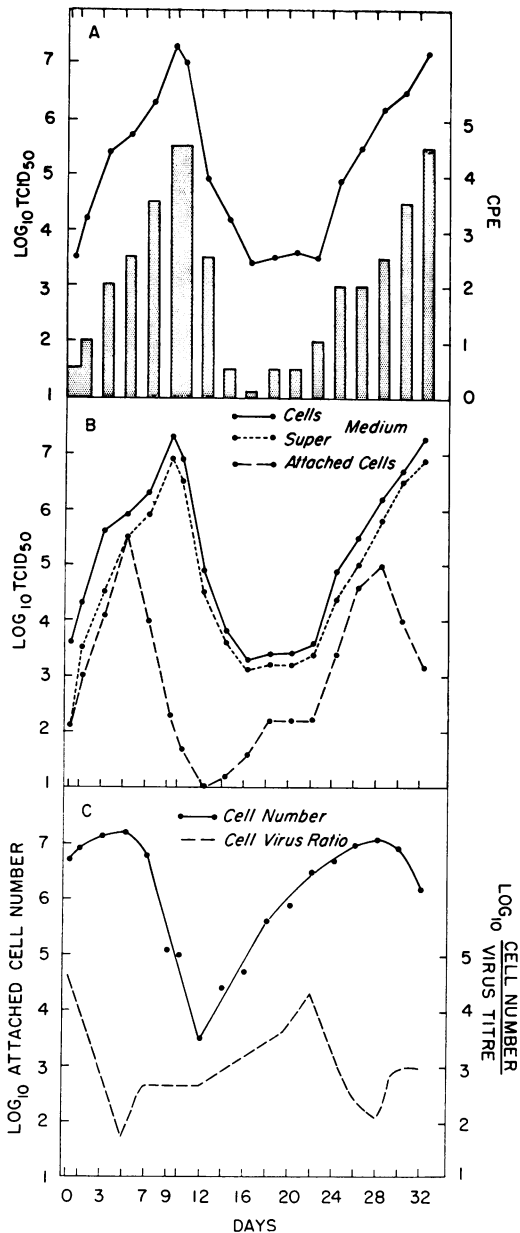
FIG. 1. *Virus synthesis and the degree of cell destruction during a cycle (see text for methods). (A) CPE (bars) and virus titers in control cultures maintained throughout the experiment to insure that the cycling patterns in the experimental cultures were parallel. (B) Virus titers in the nonsedimentable (extracellular) and sedimentable (cellular) fractions of the medium, and in the remaining attached cells. (C) Number of attached cells, and the ratio of cell number to infective virus in these cells.*

with regard to the sequence of events during the cycle. First, the percentage of attached cells remaining at the end of the cycle was less than 0.1% of the maximal cell number attained during the cycle (Fig. 1C). Second, cell proliferation preceded or paralleled synthesis of new infectious virus (Fig. 1B and 1C). Third, the appearance of infectious virus in the attached cells preceded its appearance in the medium (Fig. 1B). Finally, more virus was always present in the medium than in the attached cells, and virus in the medium was associated with both the extracellular and cellular fractions (Fig. 1B).

Susceptibility of virus-containing fractions in the medium to neutralization by antibody. The studies were designed to determine whether virus associated with the cellular and extracellular fractions of the medium was amenable to neutralization by antibody. Some variations were noted between experiments, owing to differences in the samples employed and the efficiency of cell disruption; however, the results were qualitatively reproducible. The results from two experiments are shown in Table 1. The number of intact-appearing cells per milliliter in fraction F, as determined by hemocytometer counts, was 1.82×10^6 in experiment 1 and 1.05×10^6 in experiment 2. This fraction also contained a considerable amount of cellular debris which probably resulted from the disintegration of infected cells. The total infective virus in fraction F was determined after disruption (K), and was found not to differ significantly from the non-disrupted material. Incubation with normal rabbit serum resulted in only a slight decline in the titers of the extracellular (C), nondisrupted cellular (G), and disrupted cellular (L) fractions. In contrast, treatment with anti-HSV serum resulted in almost complete neutralization of virus in the extracellular (D) and disrupted cellular (M) fractions, whereas the nondisrupted cellular (H) fraction retained approximately 10% of its infectivity which was still sedimentable at low speed (I, J). The fact that 90% or more of the infectivity in the sedimentable fraction (F) was neutralized with anti-HSV serum prior to disruption (H) indicated that this portion of the total virus was not located within intact cells, but was either on the surface of cells or associated with cell fragments. It would appear, therefore, that a maximum of 10% of the total virus in the medium resided within the intact cells. Further, since there was approximately 3 to 12 times more intact cells than total virus, the majority of these cells were probably free from infectious virus. This was studied by staining the cellular fraction with fluorescein-labeled antibody. The results obtained with fraction F from experi-

TABLE 1. *Virus titers in fractions from medium of persistently infected cultures*[a]

Fraction	Serum treatment[c]	Virus titers[b]			
		Expt 1		Expt 2	
		$TCID_{50}/ml$[d]	PFU/ml[e]	$TCID_{50}/ml$	PFU/ml
A. Original (medium)	—	$10^{5.3}$	1.4×10^5	$10^{5.3}$	3.3×10^5
B. Extracellular	—	$10^{4.5}$	1.4×10^4	$10^{4.3}$	6.1×10^4
C. Extracellular	Normal	10^4	1.1×10^4	10^4	5.4×10^4
D. Extracellular	Anti-HSV	10^0	1.0×10^0	10^0	4.0×10^0
E. Cell wash	—	10^3	1.7×10^3	10^3	2.7×10^3
F. Cellular	—	$10^{5.3}$	1.3×10^5	$10^{5.3}$	3.2×10^5
G. Cellular	Normal	$10^{5.3}$	1.2×10^5	10^5	2.6×10^5
H. Cellular	Anti-HSV	$10^{4.5}$	1.0×10^4	$10^{3.6}$	4.2×10^3
I. Cellular	Anti-HSV supernatant fluid	$10^{0.5}$	1.5×10	10^0	4.0×10^0
J. Cellular	Anti-HSV sediment	$10^{4.5}$	1.1×10^4	$1C^{3.5}$	4.4×10^3
K. Cellular (disrupted)	—	$10^{5.3}$	1.2×10^5	$10^{5.3}$	4.4×10^5
L. Cellular (disrupted)	Normal	$10^{5.3}$	1.2×10^5	10^5	3.5×10^5
M. Cellular (disrupted)	Anti-HSV	10^2	1.1×10	10^2	1.0×10

[a] Medium from persistently infected cultures refed 2 days previously was separated into various fractions as described in Materials and Methods.

[b] Virus titers in 1 ml of original medium.

[c] Treated with 10% normal rabbit serum or anti-HSV rabbit serum for 1 hr at 37 C.

[d] Determined in HeLa cells.

[e] Determined in MAL cells.

ment 1 in Table 1 indicated that only 59 of the 1,196 cells observed (approximately 5%) contained viral antigen. In contrast, it was noted that almost all of the cell fragments in this fraction fluoresced, although it was not possible to do any quantitative determinations.

Viability of the detached cells in the medium. Since the majority of the intact-appearing cells in the medium apparently did not contain infectious virus, experiments were designed to determine whether any of these cells were still viable, based on their ability to reattach to plastic and proliferate. Serial 10-fold dilutions were made in growth medium of the anti-HSV serum-treated (H) and untreated (F) sedimentable fractions, and 1-ml portions were seeded in 60-mm plastic petri dishes (Falcon Plastics, Los Angeles, Calif.) containing 3 ml of medium. Rabbit anti-HSV serum at a final concentration of 2% was added to fraction H, and the same concentration of normal rabbit serum was added to fraction F. At least two cultures per dilution were studied. The cultures were incubated at 37 C; the cells were refed three times per week, and were maintained for at least 30 days to determine the minimal cell number seeded which resulted in regrowth. The results from four separate experiments showed that regrowth occurred when plates were seeded with either anti-HSV serum-treated or untreated cells diluted to a level of 10 intact-appearing cells per

milliliter, thus indicating that at least 10% of the intact-appearing cells were viable.

Mechanisms of virus transmission. Virus associated with the extracellular and cellular fractions of the medium has been shown to be capable of transmitting infection (Table 1), including the formation of plaques in MAL cells in the presence of antibody. These results indicated that infection could be transmitted by the cell-to-cell transfer of virus, and by reinfection from the medium of virus which was susceptible to neutralization by antibody (extracellular). The finding that virus associated with the cellular fraction (F) could transmit its total infectivity in the absence of antibody (G) and approximately 10% of its infectivity in the presence of antibody (H) raised the possibility that reattachment of this cellular material to the underlying cells occurred. Experiments were designed to test this possibility. The method employed was to inoculate 0.1-ml dilutions of various fractions (Table 1) either directly on MAL cell cultures or within chambers in contact with MAL cells. The medium employed with fractions treated with anti-HSV serum was supplemented with the same antiserum to give a final concentration of 10%. The presence of normal rabbit serum in the untreated fractions did not affect the initiation of infection when compared with parallel plates from which this serum was omitted. Since the extracellular virus (B) should be able to pass

through the filter and initiate infection, while virus associated with the cellular fraction (F) should be retained within the chamber, it should be possible to demonstrate the ability of the infectious cellular material to reattach to MAL cells by comparing the titers obtained after inoculation of the various fractions either within chambers or directly on MAL cell monolayers. Table 2 shows the results from one representative experiment. The fractions employed were those from experiment 1 in Table 1. As seen in the table, the efficiency of the extracellular fraction to initiate infection was not reduced when the samples were inoculated within chambers, indicating that the extracellular virus could effectively pass through the filter and infect the underlying cells. When cultures were inoculated with the cellular fractions F or H, higher titers were observed in the absence of chambers, indicating that virus associated with the cellular material could not effectively pass through the filter. The titers obtained when samples were inoculated within chambers approximated the titers of the extracellular virus remaining in these fractions (E and I). From the results of this and similar experiments, two conclusions could be made. First, the virus associated with the cellular material must approximate the cell monolayer at a distance less than the thickness of the filter ($150~\mu \pm 10~\mu$) in order to initiate infection, and, second, the virus originally associated with the cellular material does not subsequently become eluted to enter the extracellular fluid. If this were not the case, then the titers observed when the infectious cellular material was inoculated within chambers would have approached the titers of the same samples when inoculated directly on MAL cells.

TABLE 2. *Ability of infectious cellular material from medium to reattach to MAL cell**

Fraction†	Titer in MAL cells (TCID_{50}/ml)	
	Without chamber	With chamber
B. Extracellular	$10^{4.5}$	$10^{4.5}$
F. Cellular + normal serum	$10^{5.5}$	$10^{3.5}$
H. Cellular + anti-HSV serum	$10^{4.5}$	10^{1}

* Dilutions (0.1 ml) were inoculated either within chambers which were in contact with MAL cells or directly on MAL cell monolayers. Two cultures per dilution were tested. See text for details.

† The fractions employed were from experiment 1 in Table 1.

Another series of experiments consisted of seeding 0.1-ml portions of fraction F (Table 1) on MAL cells grown on cover slips, incubating for 2 hr, washing the cover slips to remove unattached material, followed by fixation and staining with fluorescein-labeled globulins as described in Materials and Methods. Microscopic examination revealed the presence of considerable amounts of fluorescein-labeled cell fragments and a few labeled intact cells. Similar procedures with sonically disrupted and intact uninfected MAL cells as controls were negative.

It would appear, therefore, that the infectious cellular material physically contacted the underlying cells before transmission of virus occurred.

DISCUSSION

The purpose of these experiments was to further define the characteristics of these persistent infections, which is necessary before the mechanisms involved can be effectively determined. The results described in Fig. 1 graphically demonstrated the overall changes in virus titers and cell numbers which occurred during the course of one complete cycle. The severity of cell destruction is reflected in the finding that less than 0.1% of the cells were present at the end of the cycle. The absence of viral antigen in the majority of intact detached cells and their viability upon reseeding indicate that most of these cells did not contain infectious virus. This is similar to the "cell-lifting" phenomenon reported by Yerganian et al. (11) in a subline of Chinese hamster tumor cells presumably of viral etiology. The transmission of virus by the cell-to-cell and extracellular routes was similar to that reported by others for herpesviruses (2, 10). It was of interest, however, to find that virus was also transmitted by reattachment of infectious cellular material. Although the results cannot distinguish between transmission by reattachment of intact cells versus cell fragments, certain findings favor the latter mechanisms. First, most if not all of the debris stained with fluorescein-labeled antibody while only 5% or less of the intact cells were stained, and, second, treatment with antiserum prior to disruption resulted in a loss of only 90% of the infectivity. It is still possible that the viable, antigen-free detached cells may have contained virus in a noninfective form (10) which could subsequently initiate infection. Alternatively, the cells may have detached owing to an abortive infection with HSV (1), or owing to the production of a cell-detachment factor (9). The results indicated that, if transmission other than by infectious virus occurred, then the infectivity so transmitted was either a small fraction of the

total or required more than 2 weeks to become obvious. This is evident from Tables 1 and 2 where the infectivity of the nondisrupted (F) and disrupted (K) cellular fractions were similar, and the infectivity of the intact fraction F in MAL cells corresponded to the observed virus titers. Deinhardt et al. (4) reached similar conclusions in MCN cells chronically infected with various myxoviruses. However, the presumed latency of HSV in humans (3), and its reactivation after long-term cultivation of human tissues in vitro (7, 8) warrant consideration that one of the underlying mechanisms by which these cyclic persistent infections are maintained is the alternative masking and unmasking of virus.

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