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# Persistent Cyclic Herpes Simplex Virus Infection In Vitro

## III. Asynchrony in the Progression of Infection and Cell Regrowth

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ABSTRACT

HAMPAR, BERGE (National Institute of Dental Research, Bethesda, Md.). Persistent cyclic herpes simplex virus infection in vitro. III. Asynchrony in the progression of infection and cell regrowth. J. Bacteriol. **91**:1965–1970. 1966.—The progression of virus-induced cytopathic effects (CPE) and virus synthesis was studied in localized areas of Chinese hamster cell cultures persistently infected with herpes simplex virus (HSV). CPE was initially evidenced by the presence of small multinucleated giant cells, followed by expanding plaquelike lesions with an occasional uninfected cell remaining within the infected areas. Cell detachment rapidly followed the appearance of viral antigen in infected cells. The surviving cells which proliferated to re-establish the cell sheet arose from two sources. The first was from viable cells which remained attached after expansion of localized areas of CPE, and the second was from reattachment of viable cells in the medium. CPE in localized areas was initiated at various times during the cycle irrespective of the virus titer in the medium. Cell regrowth in some areas and CPE in other areas occurred simultaneously throughout the cycle in an asynchronous fashion. Consequently, during periods of rising virus titers, most areas showed CPE while few areas displayed cell regrowth. As the virus titers declined, more areas showed cell regrowth and fewer areas displayed new cycles of CPE. CPE in localized areas was not initiated until cell regrowth had occurred. It is proposed that the proliferating cells were temporarily resistant to HSV infection, and that this resistance was ultimately lost in their progeny cells.

Initial studies to characterize persistent cyclic herpes simplex virus (HSV) infections of Chinese hamster cells (3) have been reported (2). The results indicated that, during the cycles of cell destruction and regrowth, over 99.9% of the cells were destroyed or detached from the petri dish. Infectious virus was associated with the extracellular fluid, detached cellular material, and the attached cells. Finally, virus transmission occurred by reinfection with extracellular virus, the cell-to-cell transfer of virus, and by the reattachment of detached infectious cellular material.

This report continues the characterization of these infections, and is primarily concerned with determining whether cell destruction and regrowth during a cycle are separate events or whether they occur simultaneously and continuously. The results indicated that cell destruction and regrowth occurred simultaneously in different localized areas of the cultures throughout the cycling period. Evidence is also presented relating the progression of virus-induced cytopathic effects (CPE) and virus synthesis in localized areas of the cultures.

### MATERIALS AND METHODS

*Cell cultures.* The persistently infected MAL cells, tissue culture techniques, and media employed have been described (3).

*Virus titrations and fluorescent-antibody studies.* The titration of virus in HeLa cells, the recording of CPE, and the staining of cells grown on cover slips with fluorescein-labeled antibody were performed as previously described (2, 3).

*Progression of CPE.* At least five areas per culture were permanently marked by making a small scratch on the bottom of the plastic petri dish. These areas were circled, numbered, and observed daily under an inverted Zeiss Opton microscope fitted with bright-field and phase optics. Photographs were taken of the same areas at different times by use of Polaroid film

type 107 and Kodak Tri-X Pan 35 mm film. The virus titers in the medium and the degree of CPE were determined at 1- to 2-day intervals.

## RESULTS

The two techniques employed for studying the progression of CPE within localized areas were staining with fluorescein-labeled antibody and daily photography of the same areas on individual plates.

Fluorescent-antibody studies were performed after fixation of cells grown on cover slips at various times during the cycle. These experiments were designed so that the transmission of viral antigen within localized lesions could be studied in relation to the severity of CPE. At the beginning of the cycle, when a relatively intact cell sheet was present, viral antigen was restricted to a few small multinucleated cells (Fig. 1a). As CPE progressed, discrete plaquelike foci of infection formed, which expanded, leaving behind a few uninfected cells (Fig. 1b). Viral antigen was restricted to a narrow band of cells at the edge of the expanding lesions. The appearance of these progressive lesions was suggestive of the cell-to-cell transfer of virus as previously reported (2). When the CPE was maximal, masses of antigen-containing cells resulting from coalescence of adjacent infective centers were present throughout the culture (Fig. 1c). At the end of the cycle, CPE and viral antigen were restricted to a few dispersed cells or small cell masses, either in contact with or independent from uninfected cells (Fig. 1d). The relationship between cells showing CPE by microscopic observation and the presence of viral antigen was studied by comparison of the same areas under bright-field or phase microscopy and ultraviolet light. The results indicated a close relationship throughout the cycle between cells displaying viral antigen and microscopically observed CPE, and viral antigen was not evident in cells which appeared normal by microscopic examination. These same results were seen after staining the cells with Giemsa stain and observing typical HSV-induced cellular changes which were restricted to those cells displaying CPE in the unstained preparations. The rapid decline in CPE noted previously (3), along with the results of these fluorescent-antibody studies, suggested that the cells rapidly detached from the petri dish after the appearance of viral antigen. This was further evident in the finding that, throughout the cycle, the greatest amount of virus was associated with the medium, whereas the least amount of virus was found in the attached cells (2). Although the formation of small syncytia and the detachment of cells precluded quantitative determinations of the number of infected cells, the progression of CPE paralleled the increase in virus titers in the medium.

The experiments involving daily observation of the same areas by use of bright-field and phase microscopy were designed to study the progression of CPE and cell regrowth in localized areas, and to allow comparisons between different areas on the same plate. These studies had the advantage over the fluorescent-antibody studies, where the progression of CPE could not be followed in the same area. Further, the finding that microscopically evident CPE was related to the presence of viral antigen justified the use of this technique. The general appearance of the progressive lesions in localized areas was similar to that seen after fluorescent-antibody staining. The first evidence of CPE was the presence of small giant cells on a background of relatively intact cells. As the localized lesions expanded, a few normal-appearing cells usually remained behind. The CPE in the localized areas progressed until, at peak virus titers, the entire cell sheet appeared to be involved. These studies also allowed a determination of the origin of the cells which initiated regrowth after CPE. The results indicated that these cells arose from two sources. The first was regrowth from some, but not all, of the cells which remained attached after expansion of the localized lesions. Many of these cells ultimately degenerated prior to completion of successful division. The cells which were capable of successful division invariably gave rise to a colony of cells which resulted in re-establishment of the cell sheet in the localized areas. Whether the ability of these cells to survive was related to a stage in their cycle, such as previously reported for another Chinese hamster (MCH) cell system and HSV (4), is presently under investigation. The second source of regrowth was apparently from cells in the medium which reattached. This was evident from the observation that in some areas after CPE there was a complete absence of any cells. Subsequently, cells appeared within the clear areas and proliferated. These results are in agreement with the previous finding that at least 10% of the intact cells in the medium were viable, and could reattach to the plastic or to MAL cells, or both (2).

The sequence of CPE and cell regrowth in a localized area in relation to the overall cycling pattern was also studied. The results indicated that cycles of CPE and regrowth could occur in a localized area at any time during the cycle. This can be seen in Fig. 2; the overall pattern of CPE and virus synthesis in this culture is shown in Fig. 2a, and Fig. 2b–2h show the appearance of cells in one area at different times during the cycle. Figure 2b shows the initiation of CPE on day 3, which was near the peak virus titer.

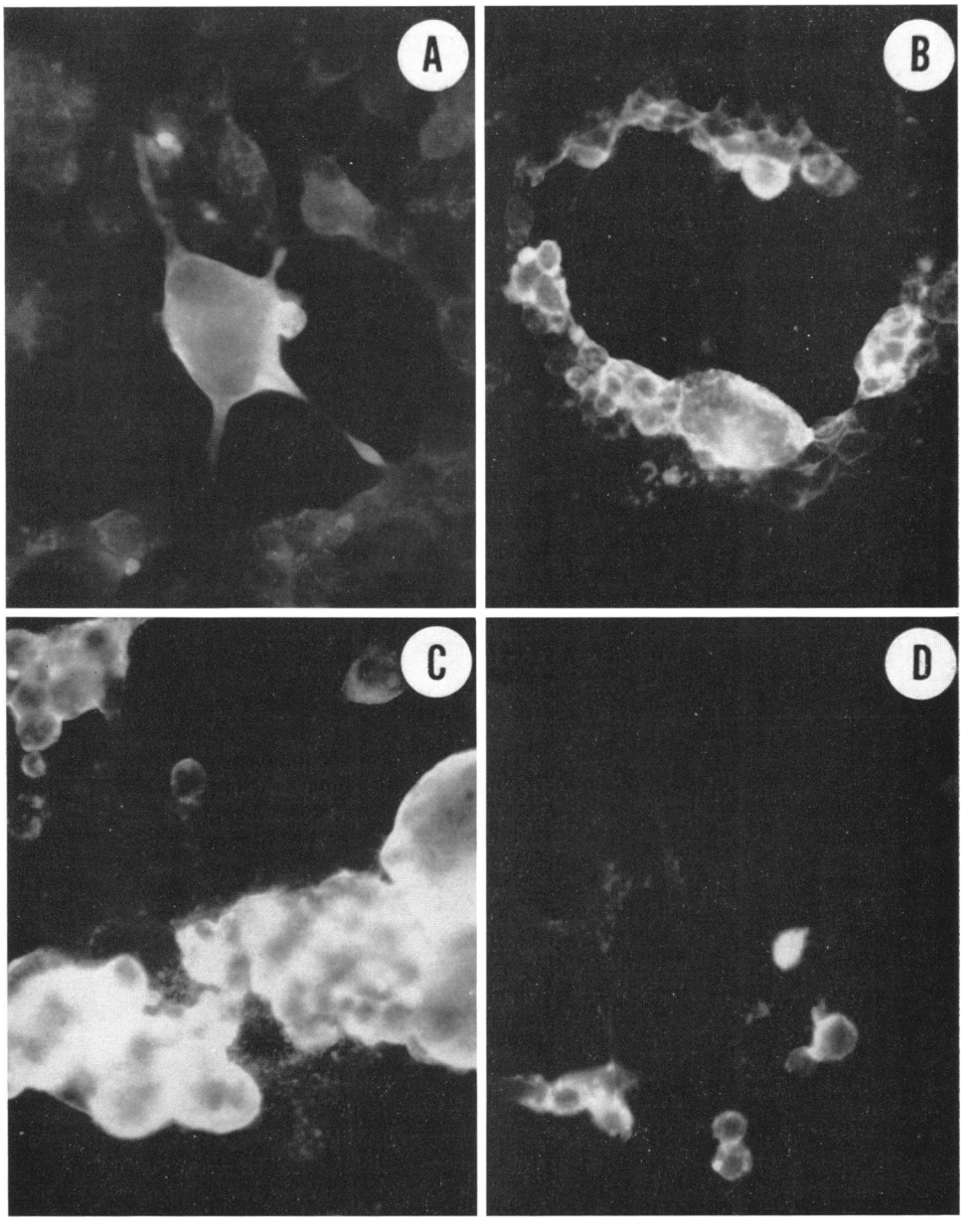


FIG. 1. *Appearance of localized viral lesions at different times in the cycle stained with fluorescein-labeled antibody. (a) Multinucleated giant cell of early lesion. (b) Expanding viral lesion with a narrow border of antigen-containing cells. (c) Large masses of antigen-containing cells present at maximal CPE in contact with underlying normal-appearing cells. (d) Dispersed HSV antigen-containing cellular material present near end of the cycle.* × *200.*

Maximal CPE was evident on day 8 (Fig. 2c), and cell regrowth was first evident on day 9 (Fig. 2d). Figures 2c and 2d are examples of cell regrowth from persisting attached cells as described above. After re-establishment of the cell sheet, CPE began again on day 18 (Fig. 2e), which was near the middle of the rise in virus titer, and progressed until day 26 (Fig. 2f), followed by regrowth of the cells (Fig. 2g). A new cycle of CPE was initiated near the end of the cycle on day 33 (Fig. 2h). It was further observed that, regardless of the virus titer in the medium and overall degree of CPE, a new cycle of infection was not initiated until a relatively intact

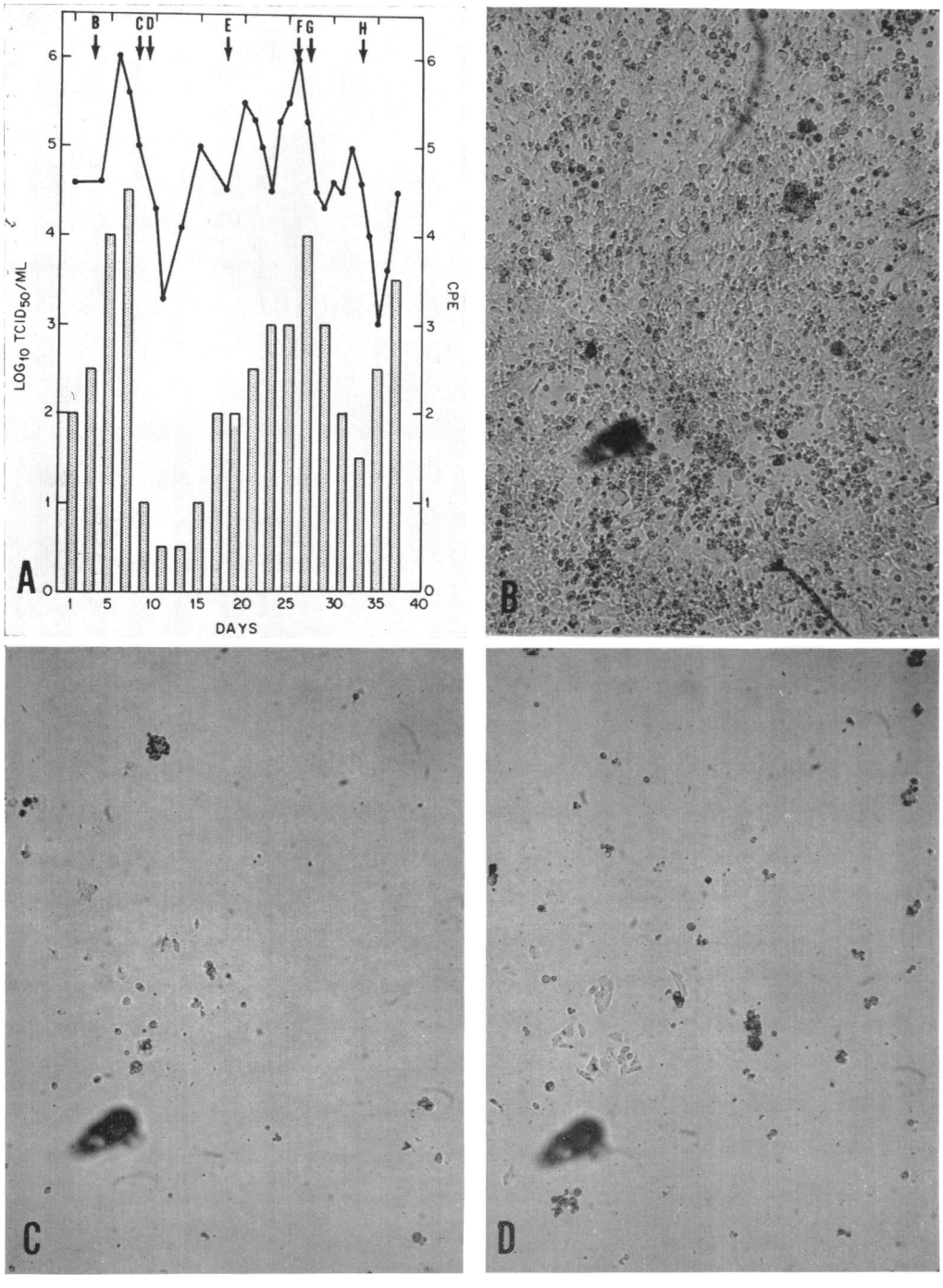


FIG. 2. *Virus titers and CPE in one culture, and the appearance of cells in one area from the same culture at various times during a cycle. (a) Virus titers in medium (solid line) and CPE (bars). The arrows refer to the days during the cycle when the succeeding photographs were taken. (b) Day 3. Initiation of CPE near period of peak virus titers. (c) Day 8. Residual cells present at maximal CPE. (d) Day 9. Early stage of cell proliferation by attached cells which survived infection. (e) Day 18. Initiation of CPE near middle of rise in virus titer. (f) Day 26. Appearance of cells at time of maximal CPE. (g) Day 27. Cell regrowth prior to initiation of new CPE. (h) Day 33. Initiation of CPE near end of the cycle.* × 31.

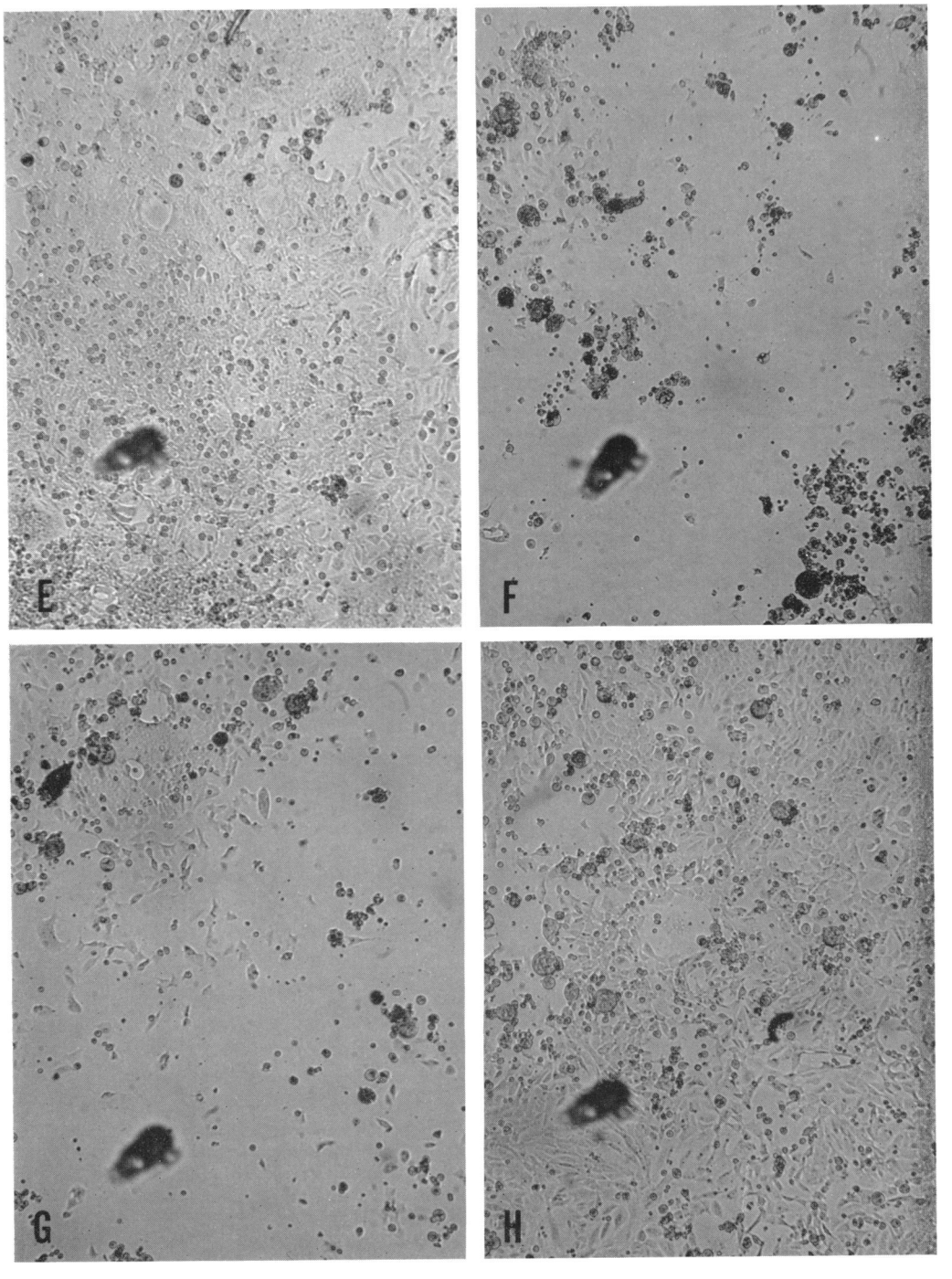


FIG. 2—*Continued*

cell sheet was re-established in a localized area (Figs. 2b, e, and h).

Finally, these studies allowed comparisons of the events in different areas of the same culture in relation to the overall pattern of virus synthesis and CPE. The results indicated that the progression of CPE and cell regrowth were asynchronous, so that, while CPE was advancing in

some areas, other areas were showing cell regrowth. This was evident in all parts of the cycle regardless of the virus titer. It was, therefore, possible to demonstrate cell regrowth during periods of maximal virus titers. These results were not unexpected since, as shown above, cycles of CPE could be initiated at any time during the cycle. Obviously, the number of areas displaying cell regrowth during the periods of rising virus titers was less than during decreasing titers, and, conversely, fewer areas showed CPE during the periods of declining titers. The overall picture of the cultures, therefore, was a relatively intact cell sheet at the end of the cycle and few remaining cells near the peak virus titers. This asynchrony in the appearance of CPE and cell regrowth in localized areas probably accounts for the step-wise increase in virus titers evident in Fig. 2a.

## DISCUSSION

The events which occur during a cycle can be summarized as follows. Infection is initiated in a few localized areas, giving rise to expanding lesions with rapid detachment of infected cells. Uninfected cells also detach (2), but the number represents a relatively minor fraction of the total cells lost. New foci of infection arise throughout the cycle by reinfection with extracellular virus and reattachment of infectious cellular material (2). Expansion of CPE progresses in these newly infected areas, while cell regrowth is occurring in areas of the original lesions. The breakdown of the cell sheet in some areas and regrowth in other areas continues throughout the cycle. This pattern follows the increase and decrease in virus titers, so that increasingly more areas display CPE during the periods of rising titers. At peak virus titers, most areas of the cell sheet show CPE and few areas display cell regrowth; consequently, the majority of the culture appears to be destroyed. This relationship is reversed during the period of declining titers when most areas display regrowth while few areas show new CPE. At the end of the cycle, therefore, a relatively intact sheet is present.

These results were not expected on the basis of previous observation of the general progression of CPE and cell regrowth in cultures or by merely counting the number of remaining attached cells at different times during the cycle (2), where the transmission of infection seemed more homogenous. One would have expected cell destruction to be limited to the early part of the cycle when the virus titers were increasing, and cell regrowth to be limited to the latter part of the cycle when the virus titers were declining. This apparently was not the case, however, and the fact that both processes were occurring simultaneously throughout the cycle should prove of importance in elucidating the mechanisms involved. In this respect, some similarities can be found with the results reported by Henle and co-workers for L-cell cultures persistently infected with myxoviruses (for a review, *see* 5). The ability of some cells to survive infection and proliferate suggests a transitory resistance (1) either to infection with HSV or to the lethal effects of previous HSV infection, which was ultimately lost in the progeny cells. The finding that re-establishment of the cell sheet in localized areas occurred prior to initiation of new cycles of CPE, although infections were simultaneously being initiated in other areas, argues against an interfering factor(s) (6) transmitted through the medium as being solely responsible for the transient resistance of these cells. If transmission through the medium was the only source of an interfering factor(s), one would have expected the protection to be equally effective in all of the cells. This was not the case, however, since the few cells remaining after the progression of CPE in localized areas underwent extensive proliferation to replenish the cell sheet prior to initiation of a new cycle of infection. The results suggest, therefore, that the temporary resistance of the cells was primarily controlled in the localized areas, and that the cells either transmitted the factor(s) responsible for resistance, or the ability to produce such a factor(s), directly to their progeny. Further, unless this factor(s) was continually being resynthesized in the localized area, it would have been rapidly diluted out and lost in the progeny cells much sooner than apparently was the case.

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