JOURNAL OF BACTERIOLOGY, Oct., 1966
Vol. 92, No. 4

Printed in U.S.A.

Production in FL Cells of Infectious and Potentially Infectious Reovirus

REX S. SPENDLOVE,[1] EDWIN H. LENNETTE, CHARLES O. KNIGHT, AND JEAN N. CHIN
Viral and Rickettsial Disease Laboratory, California State Department of Public Health, Berkeley, California

Received for publication 17 June 1966

ABSTRACT

SPENDLOVE, REX S. (California State Department of Public Health, Berkeley), EDWIN H. LENNETTE, CHARLES O. KNIGHT, AND JEAN N. CHIN. Production in FL cells of infectious and potentially infectious reovirus. J. Bacteriol. 92:1036–1040. 1966. —A comparative study was made of the development in, and release from, FL cells of infectious and potentially infectious (chymotrypsin-activatable) reovirus (Lang strain). The latent period was shorter, the rate of synthesis was more rapid, and the total yield was more than 10-fold greater in potentially infectious virus as compared with infectious virus. Almost all of the potentially infectious virus, but only approximately one-third of the infectious virus, was released from the infected cells.

Wallis, Smith, and Melnick (11) recently reported the enhancement of reovirus infectivity by treatment of infectious culture fluids with heat in the presence of 2 M $MgCl_2$. Spendlove and Schaffer (10) found the infectivity of some reovirus culture fluids to be enhanced by certain proteolytic enzymes, and that this was due to alteration of a proteinaceous material associated with the virus particles.

Although there have been several studies on the growth of infectious reovirus in mammalian cells (2, 4–6, 9), the development of enzymatically enhanceable virus, i.e., potentially infectious virus (PIV), has not been investigated. Consequently, a study of the development and release of infectious virus (IV) and PIV was undertaken, and results are presented in this report.

MATERIALS AND METHODS

Most materials and methods used in this investigation have been described in detail previously (9).

Virus. Type 1 reovirus (Lang strain) was obtained from Herbert Wenner as infectious HeLa cell culture fluid.

Cells. The FL line of human amnion cells was obtained from the Naval Biological Laboratory, Berkeley, Calif., and was passaged in a medium composed of lactalbumin hydrolysate-yeast extract, Earle's salt solution, and 10% human serum (9).

Experimental procedure. Cultures of FL cells in 3-oz prescription bottles were infected with a multiplicity of 50 to 100 infectious particles per cell with chymotrypsin-treated preparations of Lang strain of type 1 reovirus. After a 2-hr adsorption period, the cell sheets were washed twice, 4 ml of serum-free medium was added per bottle, and the cultures were incubated at 35 C. At intervals after infection, the fluids from two to five cultures were centrifuged; the cells were suspended in serum-free medium, and were frozen and thawed six times to release cell-associated virus. Samples of infected cells and extracellular fluids were assayed for infectivity by use of an immunofluorescent-cell counting technique (7) before and after chymotrypsin treatment.

Enzyme treatment. Enzymatic enhancement of reovirus infectivity has been described (10). Culture fluids containing virus were incubated for 1 hr at 37 C in the presence of 20 μg/ml of chymotrypsin.

RESULTS

Cultures sampled at various intervals after infection were assayed before and after chymotrypsin treatment to determine the titers of cell-free and cell-associated IV and PIV (Fig. 1). Infectivity assays of untreated virus suspensions enumerated IV, and assays of chymotrypsin-treated suspensions minus IV gave PIV counts.

The growth curve in Fig. 1 is representative of the five experiments performed in this investigation. The total maximal yield of cell-free and cell-associated PIV was more than 10 times higher than that of the IV. Although earlier reports (2, 4–6, 9) showed most of the IV to be retained by infected cells, most of the PIV in this study appeared to be selectively released from the cells.

The cell-free PIV appeared to acquire resistance to activation by chymotrypsin in the late stages of growth; i.e., the titer of the cell-free PIV

[1] Present address: Department of Bacteriology and Public Health, Utah State University, Logan.

dropped rapidly soon after reaching a maximal concentration. Attempts were made to determine whether the loss of enzymatic enhanceability of cell-free virus was due to thermal inactivation, to the acquisition of a resistance to enzymatic enhancement, or to susceptibility to inactivation by the enzyme.

Two groups of cell-free samples were examined from four growth experiments. The first group was collected earlier in the growth cycle and contained the maximal amount of PIV, and the second group of samples was collected 8 to 16 hr later. Untreated and enzyme-treated virus from each sample was incubated for 18 hr at 35 C.

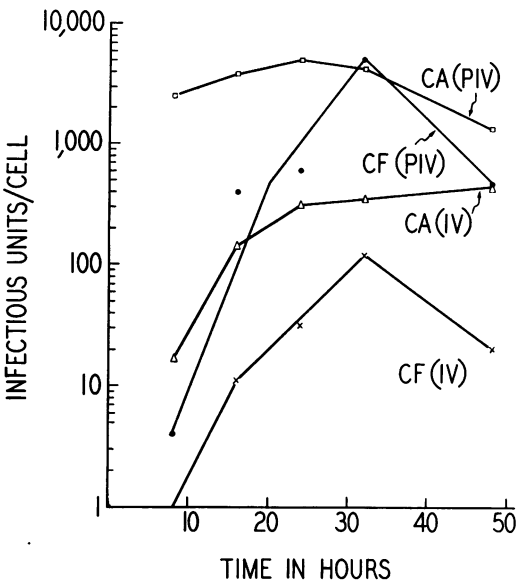

FIG. 1. *Production of infectious virus (IV) and potentially infectious virus (PIV) in reovirus-infected FL cells. CA = cell-associated; CF = cell-free.*

The infectivity titers of all samples were determined; the untreated virus was assayed before and after treatment with chymotrypsin (Table 1). Averaging the results of the four experiments showed that the infectivity of the crude (untreated) virus dropped 77% when the sample was incubated without prior enzyme treatment. The infectivity of those preparations which were enzyme-treated before incubation dropped an average of 93%. The titers of untreated virus which was treated with enzyme after incubation dropped 49%, as compared with enzyme-treated virus assayed before incubation. Thus, approximately half of the loss of infectivity was attributable to thermal inactivation.

An earlier study (10) showed enzyme-treated virus to be more thermolabile than untreated virus. This observation was confirmed in the experiments presented in Table 1. Release of proteolytic enzymes into the culture medium could result in a conversion of PIV to IV with greater thermal inactivation; such loss could conceivably be kept to low levels by periodic changes of the culture medium. When the medium on infected cultures was changed, there was an increase in the total yield of cell-free, enzymatically enhanceable virus. Figure 2 shows the results obtained with cell-free, chymotrypsin-treated virus from an experiment in which the medium on different infected cultures was changed from zero to three times. There was a typical loss of enhanceability in the unchanged control (bottom curve). The total yield of virus enhanceable by enzyme in-

TABLE 1. *Effect of incubation for 18 hr at 35 C on cell-free infectious virus (crude virus),[a] enzymatically enhanced virus, and potentially infectious virus (enzyme-enhanceable virus)*

Virus	Growth expt sample					
	Maximum			Postmaximum		
	Before incubation	After incubation	Survivors	Before incubation	After incubation	Survivors
			%			%
Crude virus[b]	24[e]	6.2	26	6.5	1.3	20
Enzyme-enhanced virus[c]	380	23	6.1	96	7	7.3
Enzyme-enhanceable virus[d]	380	210	55	96	46	48

[a] Virus not chymotrypsin-treated.
[b] Thermostability of crude virus.
[c] Thermostability of enzyme-enhanced virus.
[d] Thermostability (enhanceability) of crude virus enzyme treated after incubation.
[e] Each figure is the average of results obtained in four experiments. Maximal samples contained the maximal concentration of cell-free virus (infectious virus plus potentially infectious virus), and were taken between 24 and 32 hr postinfection; postmaximal samples were taken between 32 and 48 hr postinfection. Results (except per cent survivors) expressed as infectious virus per milliliter ($\times 10^7$).

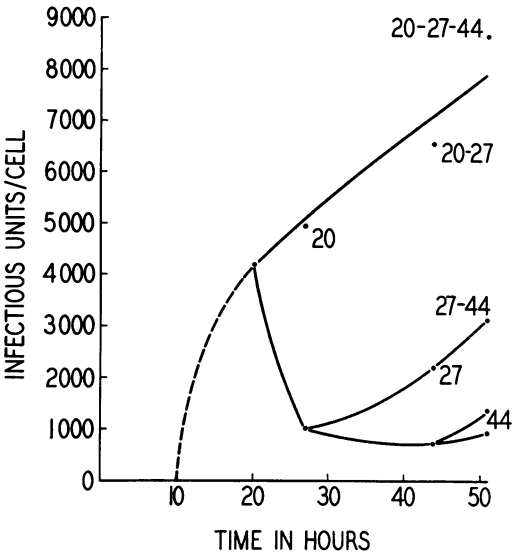

FIG. 2. *Effect of medium changes on the accumulated yield of potentially infectious reovirus released from infected FL cells. The numbers on the graph indicate the time(s) of previous medium changes. The cultures from which the results were obtained for the lower curve were sampled, treated with chymotrypsin, and assayed without previous medium changes.*

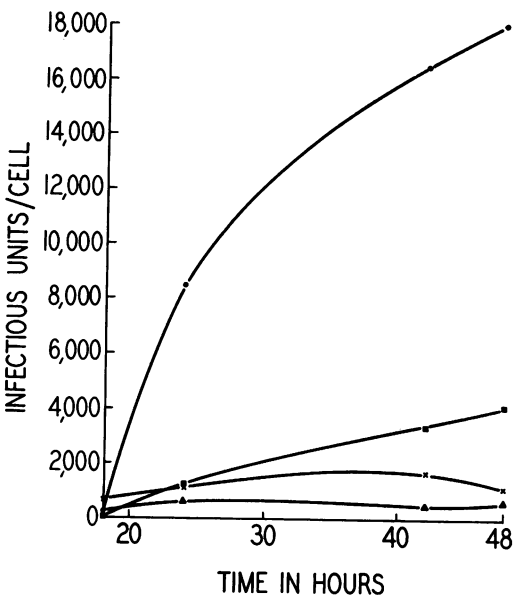

FIG. 3. *Effect of medium changes and cell density on the accumulated yield of potentially infectious reovirus released from infected FL cells. Symbols: ● and ■ represent a cell density of 8.0 × 10⁵ and 1.7 × 10⁶ cells per milliliter, respectively; the medium on these cultures was removed, sampled for assay, and replaced with fresh medium. The plotted points are a sum of the assays of all culture mediums removed to the indicated time interval. The symbols × and ▲ represent a cell density of 8.0 × 10⁵ and 1.7 × 10⁶ cells per milliliter, respectively; the medium on these cultures was sampled for assay without medium changes. The plotted points indicate the titer of the sample.*

creased as the number of culture fluid changes was increased.

After 51 hr of infection, the accumulated yield of cell-free PIV from the various cultures was as follows: 950 infectious units per cell from the unchanged cultures, 1,350 from the cultures changed once at 44 hr, 3,100 from the cultures changed at 27 and 44 hr, and 8,660 from those changed at 20, 27, and 44 hr.

The above results indicate that the cell concentration should be an important factor in determining the number of medium changes required for optimal virus yields. Figure 3 shows the results obtained when cultures of two different cell densities were infected and harvested with and without periodic medium replacements. After 48 hr of infection, monolayer cultures containing 8.0×10^5 cells per milliliter yielded 18,100 infectious units of PIV per cell when the culture medium was changed at 18, 24, and 42 hr postinfection. Enzyme-treated fluids from cultures of the same cell density sampled without medium changes yielded only 1,100 infectious units per cell. When supernatant fluids from monolayer cultures having a cell density of 1.7×10^6 cells per milliliter were treated with enzyme, an accumulated yield of 4,100 infectious units of virus was obtained per cell when the medium was replaced three times during infection. Only 680 infectious units of virus was released per cell when the cultures were sampled at 48 hr postinfection without previous changes of the medium.

DISCUSSION

The characteristics of reovirus growth which have been reported previously (2, 4–6, 9) are: (i) a latent period of 6 to 9 hr; (ii) a slow intracytoplasmic development of infectious virus (the maximal concentration of cell-associated virus appeared between 15 and 54 hr in the various studies); (iii) a limited release of infectious type 1 and 3 virus (only 7 to 22% of the total infectious virus was released), and a 55% release of reovirus type 2 from infected cells (4); and (iv) a total yield of infectious virus particles between 225 and 2,600 per cell.

In the above studies of reovirus growth, no information was obtained on the development of PIV. When total virus (IV and PIV) development and release are considered, the growth of the Lang strain in, and release from, FL cells differs

considerably from earlier growth studies of IV of the three different serological types.

Isaacs (3) showed, by use of six examples, that ribonucleic acid (RNA) viruses in general have a shorter lag period and a faster rate of increase than deoxyribonucleic acid (DNA) viruses. Gomatos et al. (2) added several other examples to Isaacs' list, and noted that reovirus is an unusual RNA virus in that its multiplication is relatively slow. Since the reoviruses contain double-stranded RNA, it would not be surprising to find their growth to be slow and more like that of double-stranded DNA viruses. However, the production of IV plus PIV in cells infected with the Lang strain of reovirus is comparable with the rate of growth of most other RNA viruses.

Isaacs (3) pointed out that a rate of increase of infectivity of the order of about 1 $\log_{10}$ per hour has been reported for poliovirus, Western equine encephalitis virus, influenza virus, and Newcastle disease virus. The reported yields of infective doses per cell with the respective viruses were 100, 200 to 1,000, 650, and 1,000. These reports were of early studies, and the yields are certainly not maximal for these viruses; however, the figures can be used in comparison to show that the latent period for reovirus is not long, the rate of development is not slow, and the final yield of virus is not low.

In the present studies, a short latent period was indicated. Although early samples were not taken, it seems unlikely that the latent period extended for 6 hr, the shortest latent period reported earlier, because more than 2,400 PIV particles were produced by 8 hr postinoculation. This quantity of virus at such an early hour suggests a rate of synthesis equal to, or greater than, 1 $\log_{10}$ per hour, or a latent period considerably less than 6 hr. If one assumes a 1 $\log_{10}$ increase per hour as found with several other RNA viruses (3), then the cell-associated PIV extrapolates back to a latent period of approximately 4.5 hr. Further studies on this aspect of reovirus growth are anticipated.

The maximal reovirus accumulation (cell-free and cell-associated IV plus PIV) exceeded 10,000 infectious units per cell at 32 hr postinoculation, although the maximal concentration of cell-associated PIV was reached relatively late in the infection (between 16 and 32 hr postinfection); this was not because of a slow rate of PIV synthesis, but was probably because of limited host-cell damage with continued virus synthesis. An accumulated yield of over 18,000 IV plus PIV particles per cell was obtained in one experiment, in which the cell density was reduced and the medium on the infected cells was replaced several times.

An interesting aspect of virus release was that PIV appeared to be selectively released and the IV selectively retained, or that both were released but the IV was thermally inactivated at a much more rapid rate than the PIV. This was evident in three observations: (i) the peak concentration of cell-associated PIV always appeared at an earlier time than that of the IV, i.e., the cell-associated PIV appeared to be released and did not continue to accumulate; (ii) as the rate of increase of the cell-associated PIV began to decline, there was a corresponding increase in the cell-free PIV (this was not as evident with the IV); and (iii) the maximal concentration of cell-free PIV was always equal to, or greater than, that of the cell-associated PIV, whereas the maximal concentration of cell-free IV was always much less than the maximal cell-associated IV.

A better explanation of the development and release of PIV awaits the elucidation of the relationship of PIV to IV. A previous study (10) showed that the enzymatic enhancement of reovirus infectivity was due to proteolytic action upon a virus-associated substrate. It was not determined whether the substrate was a bound inhibitor or some portion of the virus coat. The enhancement did not appear to be due to disaggregation of clumps of virus, since hemagglutinating activity of the virus was not enhanced.

The reoviruses have been shown to associate themselves with the spindles of the mitotic apparatus (1, 7, 8); however, it is unlikely that the inhibition is due to attachment of virus to spindle tubules. This would require a selective release of spindle tubule material with accompanying virus from the cell and retention of virus that was not associated with the spindle, or association of virus with the spindle would have to prevent the virus from becoming inhibited and also from being released.

In this investigation, the titer of the cell-free virus decreased in the later stages of the growth period. A logical explanation would be that cellular proteolytic enzymes were acting on the released virus, making it more susceptible to thermal inactivation. Whatever the mechanism, the knowledge that loss of enhanceability occurs can be applied in the production of high-titered virus stocks. Nonenhanceability can be minimized by periodic changes of the medium on infected cells, and also by maintaining a relatively low cell density in the infected cultures.

The early studies on reovirus growth (2, 4–6, 9) indicated that little infectious virus was released from infected cells. If the results obtained with FL cells infected with the Lang strain of reovirus are indicative of what might be expected with other reovirus strains in various host cells, then

it would seem advisable to make certain that large quantities of released PIV are not discarded in supernatant fluids.

ACKNOWLEDGMENTS

We wish to thank Mary E. McClain for her advice and criticisms in the preparation of this manuscript.

This investigation was supported by Public Health Service grant AI-01475 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. DALES, E. 1963. Association between the spindle apparatus and reovirus. Proc. Natl. Acad. Sci. U.S. **50**:268–275.
2. GOMATOS, P. J., I. TAMM, S. DALES, AND R. M. FRANKLIN. 1962. Reovirus type 3: physical characteristics and interaction with L cells. Virology **17**:441–454.
3. ISAACS, A. 1959. Biological aspects of intracellular stages of virus growth, p. 111–156. *In* F. M. Burnet and W. M. Stanley [ed.], The viruses, vol. 3. Academic Press, Inc., New York.
4. OIE, H., P. C. LOH, AND M. SOERGEL. 1966. Growth characteristics and immunocytochemical studies of reovirus type 2 in a line of human amnion cells. Arch. Ges. Virusforsch. **18**:16–24.
5. RHIM, J. S., L. E. JORDAN, AND H. D. MAYOR. 1962. Cytochemical, fluorescent-antibody and electron microscopic studies on the growth of reovirus (ECHO 10) in tissue culture. Virology **17**:342–355.
6. RHIM, J. S., K. O. SMITH, AND J. L. MELNICK. 1961. Complete and coreless forms of reovirus (ECHO 10). Virology **15**:428–435.
7. SPENDLOVE, R. S., E. H. LENNETTE, J. N. CHIN, AND C. O. KNIGHT. 1964. Effect of antimitotic agents on intracellular reovirus antigen. Cancer Res. **24**:1826–1833.
8. SPENDLOVE, R. S., E. H. LENNETTE, AND A. C. JOHN. 1963. The role of the mitotic apparatus in the intracellular location of reovirus antigen. J. Immunol. **90**:554–560.
9. SPENDLOVE, R. S., E. H. LENNETTE, C. O. KNIGHT, AND J. N. CHIN. 1963. Development of viral antigen and infectious virus in HeLa cells infected with reovirus. J. Immunol. **90**:548–553.
10. SPENDLOVE, R. S., AND F. L. SCHAFFER. 1965. Enzymatic enhancement of infectivity of reovirus. J. Bacteriol. **89**:597–602.
11. WALLIS, C., K. O. SMITH, AND J. L. MELNICK. 1964. Reovirus activation by heating and inactivation by cooling in $MgCl_2$ solutions. Virology **22**:608–619.