Inhibition of Synchronized Cellular Deoxyribonucleic Acid Synthesis During Newcastle Disease Virus, Mengovirus, or Reovirus Infection

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Cultures of L cells were synchronized with respect to deoxyribonucleic acid (DNA) synthesis with thymidine and 5-fluoro-2'-deoxyuridine (FUdR) and infected with Newcastle disease virus (NDV), mengovirus, or reovirus 3. Inhibition of incorporation of ^{3}H-cytidine into the DNA of synchronized cells is partially inhibited 2 hr after infection with NDV or mengovirus and nearly completely suppressed 4 hr after infection. With NDV and mengovirus, no evidence was obtained of differences in sensitivity of cells during early S phase as compared to later stages in DNA synthesis. When cells were infected with reovirus at the time of release from FUdR block, inhibition of cellular DNA synthesis was evident at 2 to 3 hr, and it was complete at 4 to 5 hr after infection. However, when cells were infected several hours prerelease, synthesis of DNA occurred in early S phase in spite of the fact that the cells had been infected for up to 6 hr. The results indicate that DNA synthesis in early S phase is relatively insensitive to the inhibitory function of reovirus. Colorimetric determinations (diphenylamine reaction) of the amounts of DNA produced in synchronized cells have substantiated the inhibition of DNA synthesis observed by isotope incorporation techniques.

Newcastle disease virus (NDV) (12), mengovirus (6), and reovirus infections (8) all cause inhibition of the synthesis of cellular deoxyribonucleic acid (DNA). NDV and mengovirus also inhibit the synthesis of cellular proteins, and it appears that the inhibition of cellular DNA synthesis in these cases is secondary to inhibition of cellular protein synthesis (5). In contrast, no reduction in overall protein synthesis is observed in reovirus-infected cells (3, 8). Available evidence supports the view that inhibition of DNA synthesis in reovirus-infected cells may be a direct effect.

In all these infections, the inhibition in cellular DNA synthesis is associated with an apparent reduction in the number of chromosomal regions active in replication, rather than a reduced rate of polymerization within such regions (4, 5). No changes have been demonstrated in the physical state of the cellular DNA template or the level of activity of selected enzymes in the DNA synthetic pathway in cells infected with NDV, mengo, or reovirus. Also, the time course of the growth of daughter DNA chains in infected cells is similar to that in uninfected cells. However, the number of growing daughter chains appears to be reduced,

which has suggested that in all cases inhibition is due to a block of initiation of DNA synthesis upon independently replicated regions of chromosomal DNA (4, 5).

To obtain further evidence concerning the mechanism of inhibition of cellular DNA synthesis in these virus infections, it was decided to employ synchronized cultures of cells (7, 9, 11). Use of synchronized cultures allows investigation of the sensitivity of DNA replication to inhibition during various parts of the DNA synthetic phase. Moreover, DNA synthesis can be monitored in synchronized cells by colorimetric techniques, whereas in unsynchronized cells this is made difficult by a large background of nonreplicating DNA.

MATERIALS AND METHODS

L cells were maintained in suspension culture as previously described (3). The production of NDV, mengovirus, and reovirus type 3 stocks and the growth characteristics of these viruses in suspension cultures of L cells have been previously described (3–5). Cells were synchronized with respect to DNA synthesis by the combined use of thymidine and 5-fluoro-2'-deoxyuridine (FUdR). Cells at 10^5 to 2×10^5 cells/ml were first blocked with 2.0 mm

thymidine (added as a 50X concentrated solution) for 14 hr. The cells were sedimented in a warm centrifuge, suspended, and centrifuged in warm, fresh growth medium which lacked thymidine. The pelleted cells were resuspended in fresh medium at 10^5 to 2×10^5 cells/ml. After 8 hr, FUdR was added to a final concentration of 2 μM (added as a 100X concentrated solution). Cells were released from the second block 12 to 14 hr later by the addition of thymidine to a final concentration of 20 μM (added as a 100 X concentrated solution). Two such consecutive, appropriately-timed blocks, separated by a period of release, were used to accumulate essentially all cells at the beginning of S phase. Cumulative incorporation of cytidine-5-^3H was used to measure DNA synthesis. Three 5-ml samples of labeled cells were analyzed at appropriate times after release. Labeled cytidine incorporated into ribonucleic acid (RNA) was removed by digestion at 37 C in 0.3 N KOH for 12 hr (10). Acid-precipitable radioactivity in DNA was measured as previously described (5). Chemical determinations of the amount of DNA present in samples of cells were carried out using the Burton modification of the diphenylamine reaction (1). Salmon sperm DNA was used as the standard. Thymidine (TdR) was purchased from P-L Biochemicals, Milwaukee, Wis.. 5-Fluoro-2'-deoxyuridine (FUdR) was made available by W. E. Scott of Hoffman-La Roche Inc., Nutley, N. J. Cytidine-5-^3H (24 c/mmole) was obtained from Amersham/Searle Co., Des Plaines, Ill. Salmon sperm DNA was obtained from Worthington Biochemical Corp., Freehold, N. J.

RESULTS

Figure 1 shows that the length of the DNA synthetic phase in uninfected control cells was approximately 6 hr. The increase in DNA was essentially linear over this period. In the experiment shown, NDV or mengovirus was added 1 hr after release of cells from FUdR block. Inhibition of cellular DNA synthesis is evident in infected cells by 2 hr after infection, i.e., by 3 hr after release, and becomes increasingly more complete so that, after the 4th hr of infection, there is very little additional incorporation of labeled cytidine into DNA.

It is evident that infection with NDV or mengovirus leads to cessation of cellular DNA synthesis, and not merely to a reduction in the rate of synthesis. In other experiments, in which cells were infected at release, cellular DNA synthesis was also inhibited by 2 hr after infection and almost completely inhibited by 4 hr after infection. The interval between appearance of inhibition and cessation of DNA synthesis is thus approximately 3 hr. This interval may at least in part reflect the rate of accumulation of viral products in the infected cell.

There are two possible explanations as to why there was no inhibition during the early hours
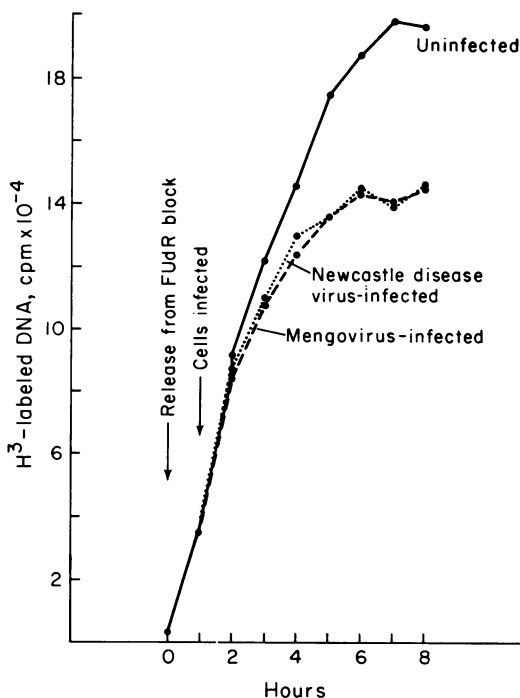

FIG. 1. *DNA synthesis in synchronized cultures of L cells infected with Newcastle disease or mengovirus. One hour after release from the FUdR block, cells were inoculated with Newcastle disease virus or mengovirus at an input multiplicity of 200 plaque-forming units/ cell. Cumulative incorporation of ^3H-cytidine (2 μc/ml) into DNA was measured.*

after infection. It may take the infecting virus at least 1 hr to express the function which is responsible for inhibition of cellular DNA synthesis; or, alternatively, DNA synthesis may not be sensitive to viral effects during the 1st hr after release from block. To decide between these alternatives, experiments were done in which cells were infected with NDV or mengovirus 3 hr prerelease. There was little or no DNA synthesized after release under these circumstances. These results indicate that DNA synthesis early in S phase is sensitive to inhibition by infection with NDV or mengovirus, but that it takes these viruses at least 1 hr to express inhibitory functions.

Figure 2A shows the time course of synthesis of DNA in cells infected with reovirus at the time of release from FUdR block. Inhibition of cellular DNA synthesis is evident 2 to 3 hr after infection and release. Synthesis of cellular DNA in infected cells stops within 4 hr after infection and release. In several experiments of this type, the synthesis of cellular DNA has ceased by the 4th or 5th hr after infection. These results indicate
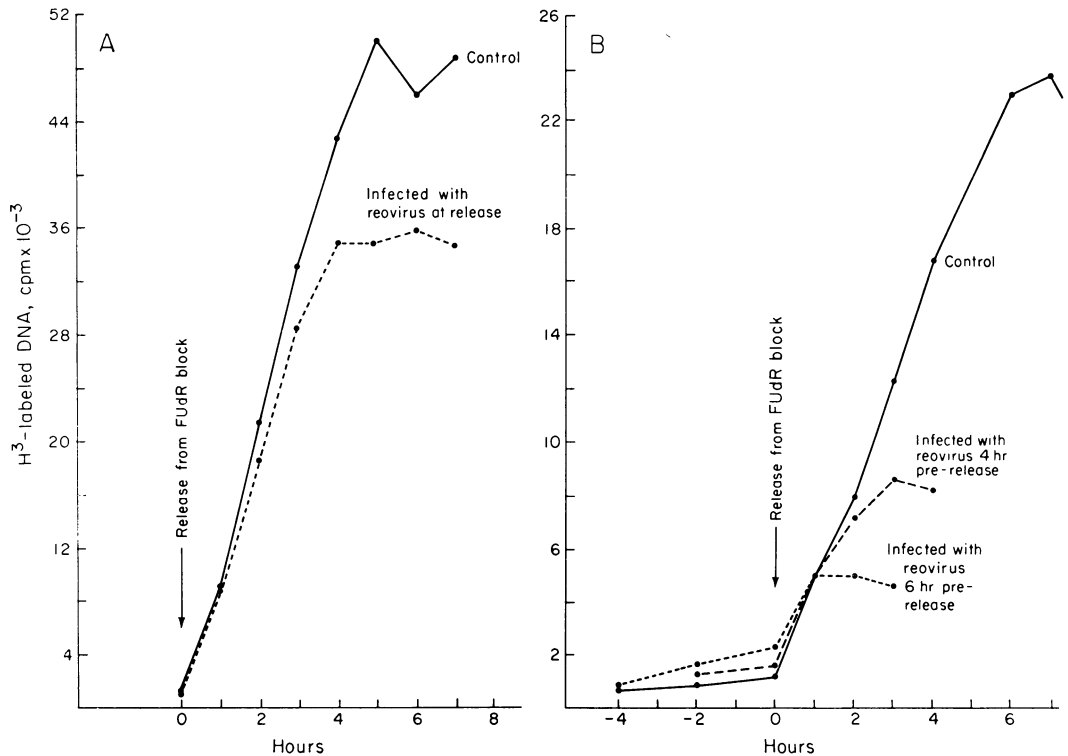
Fig. 2. *DNA synthesis in synchronized cultures of L cells infected with reovirus. Cells were inoculated with reovirus at an input multiplicity of 100 plaque-forming units/cell. Cumulative incorporation of ^3H-cytidine (2 $\mu c/ml$) into DNA was measured. A, Cells infected at the time of release from the FUdR block. B, Cells infected either 6 or 4 hr prerelease from the FUdR block.*

that the inhibitory function of reovirus on cellular DNA synthesis is expressed by 2 to 3 hr after infection. Also, it appears that approximately 3 hr are required from earliest detectable inhibition until complete cessation of DNA replication. It is clear that only a part of the total DNA complement of the synchronized cells is replicated after infection.

When cells were infected with reovirus several hours before release, evidence was obtained that DNA synthesis in early S phase is relatively insensitive to the inhibitory function of reovirus. The results summarized in Fig. 2B show that cells infected 6 hr prior to release could synthesize a significant amount of DNA during the 1st hr after release, but not later. Cells infected 4 hr prior to release incorporated near normal amounts of radioactive precursor into DNA for the first 2 hr after release, but there was little incorporation thereafter. Thus, cells infected several hours prerelease are able to synthesize unexpectedly large quantities of DNA during the period immediately following release. After prerelease infection, DNA replicates in early S phase

cells as late as 5 to 7 hr after infection, but replication does stop abruptly thereafter. As reported above, DNA synthesis ceases 4 to 5 hr after infection in cells infected with reovirus at release from FUdR block.

In all of the experiments described, DNA synthesis ceases before significant morphological evidence of cell damage becomes apparent in the synchronized infected cells, at approximately 8 hr after infection with reovirus, when damage also is noted in unsynchronized infected cells in suspension culture (3). When synchronized cells were infected with reovirus 9 hr prior to release, no synthesis of cellular DNA occurred after release. In view of the morphological evidence of cell damage at 9 hr and later, it is possible that DNA synthesis was prevented due to nonspecific causes.

The results of direct chemical determinations of the amounts of DNA synthesized after release in NDV-, mengovirus-, or reovirus-infected cells (Table 1) are compatible with the decreased incorporation of radioactive precursors into DNA.

TABLE 1. *Inhibition of L-cell DNA production during NDV, mengovirus, or reovirus infection*[a]

Synchronized L cells[b]	DNA increase during 6 hr after release (μg/5 $\times$ 10^6 cells)	DNA increase in infected cells (% of uninfected control)
Uninfected...............	75[c]	
Infected with NDV at time of release..............	35	47
Infected with mengovirus at time of release.......	30	40
Uninfected...............	96[d]	
Infected with reovirus at time of release.........	63	66
Infected with reovirus 2 hr before release.........	40	42

[a] Amounts of DNA were measured by the diphenylamine reaction.

[b] Released from the FUdR block with thymidine. Input multiplicity of NDV and mengovirus: 200 plaque-forming units/cell; reovirus: 100 plaque-forming units/cell.

[c] Cells (5 $\times$ 10^6) contained 110 μg of DNA prior to release.

[d] Cells (5 $\times$ 10^6) contained 100 μg of DNA prior to release.

DISCUSSION

The present results show that NDV or mengovirus infection inhibits L-cell DNA synthesis in synchronized cells with kinetics which are similar to those described for unsynchronized cells (5). In contrast, infection of synchronized cells at release with reovirus causes inhibition of DNA synthesis significantly sooner than observed in unsynchronized cells infected under similar conditions (3, 4). An explanation of this finding is suggested by the observation that DNA synthesis in cells in early S phase is not affected as much by reovirus infection as it is in cells in later stages of the S phase. Since cells in an unsynchronized culture are distributed through the cell cycle, the composite kinetics of reovirus-induced inhibition of DNA synthesis in unsynchronized cells reflects an average of early and later S phase inhibitory effects. The expected result would be that found experimentally, namely a delay in the detection of reovirus-induced inhibition of DNA synthesis in unsynchronized cultures as compared to synchronized cultures. Note that DNA synthesis in early S-phase cells is sensitive to inhibition by NDV or mengovirus infection, and the time when inhibition of DNA synthesis is detected is similar in synchronized and unsynchronized cells.

The findings with reovirus raise the possibility that, at the beginning of the S phase, DNA replicates under conditions that are different from those which apply later. Previous autoradiographic experiments in unsynchronized cultures have demonstrated (7) that reovirus infection does not reduce the number of cells with nuclear grains, but it does reduce the amount of labeled thymidine incorporated per nucleus. Thus, both the previous and present results indicate that a reovirus-infected cell is able to enter DNA synthesis but that infection causes a rapid decline in the amount of DNA replicated.

Recent studies on heteroploid human amnion cells synchronized with excess thymidine and amethopterin have shown that, during the first 10 min of the S period, incorporation of ^{3}H-thymidine label is restricted to the periphery of the nucleus and nucleolus (2). In synchronized cells labeled for 20 min the distribution is more homogeneous. In unsynchronized cells labeled for 10 min, the grains are not restricted to the periphery of the nucleus but are homogeneously distributed in the nucleus. These observations have suggested that DNA synthesis in a cell begins at the nuclear membrane and have focused attention on the functional importance of attachment of chromosomes to nuclear membrane. The importance of these results for studies with reovirus is that they point to a distinction between DNA replication during the earliest part of the S period and subsequent DNA replication.

An attractive hypothesis is that reovirus infection has no effect on that DNA replication which takes place in association with the nuclear membrane in early S phase. Save for the lack of thymidine, the FUdR-blocked cell is ready to enter the S phase. It may be postulated that the necessary association of DNA template, enzyme(s), initiator(s), and nuclear membrane may have taken place many hours prior to release from block, possibly even before infection with virus. Under these conditions there apparently is no target in early S phase for the highly selective inhibitory function of reovirus, although NDV and mengovirus still find a target, which may be the synthesis of some additional necessary protein.

The mechanism by which, in later parts of S phase, reovirus infection apparently blocks initiation of daughter chains upon new regions of DNA is an as yet unsolved problem (4). The new results have underlined the selectivity of the inhibitory effect of reovirus infection on cellular DNA replication.

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