System of Double Infection Between Vaccinia Virus and Mengovirus

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When L cells are simultaneously infected with vaccinia virus and mengovirus, double interference in the replication of both viruses is observed. Superinfection of vaccinia virus-infected cells by mengovirus during the first 5 hr of infection reduces vaccinia virus yields to between 1 and 3% of controls. The yields of mengovirus are reduced to between 1 and 16% of controls, depending upon the time of superinfection. The replication of vaccinia deoxyribonucleic acid is not inhibited by mengovirus; it is only delayed. On the other hand, vaccinia multiplication severely hinders the replication of mengovirus ribonucleic acid. The double-infected system, at early times, synthesizes proteins that resemble those synthesized in the vaccinia virus-infected cells. Later in infection, however, the pattern is switched to proteins synthesized by mengovirus-infected cells. Possible mechanisms for this double interference in multiplication are discussed.

A system of double infection utilizing vaccinia virus and mengovirus was studied in an attempt to elucidate the mechanism by which viruses control ribonucleic acid (RNA) and protein synthesis. The question was posed as to whether the inhibition of macromolecular synthesis is a specific effect directed against host macromolecular synthesis or whether these viruses inhibit all macromolecular synthesis except their own.

Mengovirus infection results in the inhibition of host RNA and deoxyribonucleic acid (DNA) synthesis (22). We asked whether mengovirus inhibits only host RNA or whether this inhibition also affects other kinds of RNA synthesized by the system. Furthermore, it has not been clarified whether inhibition of host protein synthesis by these viruses is an indirect effect or whether there is a direct protein inhibitor, coded or triggered by the invading virus.

Systems of double infection have been described previously. In double infection between vaccinia virus and reovirus, it was observed that reovirus production is greatly reduced (5). When cells were double-infected with poliovirus and simian virus 5, no interference with simian virus 5 production was observed (3). It is known that guanidine-inhibited poliovirus permits the synthesis of mengovirus RNA and protein (18). Cords and Holland (4) reported that, although simultaneous infection of a cell with two enteroviruses resulted in normal production of infectious RNA of both viruses, delayed infection by one of the enteroviruses resulted in a drastic inhibition of the production of infectious RNA of the superinfecting virus. More recently, it was reported that frog virus 3 inhibits the replication of vaccinia virus DNA (1). This was dependent upon the multiplicity of infection with frog virus 3.

Similarly, Giorno and Kates (7) have shown that vaccinia virus is unable to replicate in HeLa cells preinfected for 18 hr with adenovirus type 2. In this case, vaccinia virus RNA synthesis occurred, but the RNA failed to associate with the polysomes of the cell. Vaccinia virus DNA synthesis did not occur under these conditions.

The system of double infection between vaccinia virus and mengovirus is of particular interest, since it is possible to study the interaction of a DNA and an RNA virus, both of which multiply in the cytoplasm of the cell.

MATERIALS AND METHODS

Cells and viruses. L cells (mouse fibroblasts) were derived from stocks of John Littlefield of the Massachusetts General Hospital, Boston, Mass., and were grown in Spinner culture in Eagle's medium (6) as modified by Joklik and supplemented with 10% fetal calf serum.

Vaccinia virus stocks were prepared by infecting mouse L cells in Spinner culture as described by Joklik (12). After 3 days, cells were harvested and
disrupted by sonic treatment, and the remaining cell debris was removed by low-speed centrifugation. Mengovirus was kindly supplied by John Holland, University of California, La Jolla, Calif. Stocks were prepared by infecting L cells grown in Eagle's medium (6) as monolayer cultures. After 2 days, cells were harvested and disrupted by sonic oscillation. With these procedures, vaccinia virus stocks obtained had a titer of approximately 10^8 plaque-forming units (PFU)/ml while mengovirus titers were of the order of 10^6 PFU/ml.

Vaccinia virus was assayed on monolayers on chick embryo fibroblasts (CEF; reference 23). Fibroblasts that were grown in Eagle's medium supplemented with 5% horse serum were infected and overlaid with medium containing 1% agar. Plaques were counted 4 days after infection.

Mengovirus was assayed on L-cell monolayers grown on Eagle's medium with 5% calf serum and, after infection, overlaid with 1.0% washed agar containing the same supplemented medium (2). Plaques were counted 72 hr after infection.

To determine whether there was interference of assay of one virus in the presence of the other, mixtures of both viruses (in different proportions) were prepared and assayed. The results are shown in Table 1. When a sample containing only vaccinia virus particles was plated on L-cell monolayers, very small plaques plated at 0.1% efficiency compared to that in CEF were detected. These plaques were easily distinguished from the much larger mengovirus plaques on L cells. No appreciable interference was observed within the concentration ranges used in this work. No plaques were detected when mengovirus was plated on CEF monolayers.

All experiments were performed by using L cells growing in Spinner cultures. Suspensions of 10^6 cells/ml were infected with vaccinia virus at a multiplicity of 5 PFU/cell. After incubation at 37 C for 1 hr, the cells were washed three times in complete medium and diluted to 5 × 10^6 cells/ml. The cells were then superinfected with mengovirus at a multiplicity of 100 PFU/cell. If the yields of mengovirus were to be determined, the cells were again washed with complete medium 1 hr after mengovirus infection. For simultaneous infections, the concentrated cell suspension was infected with both viruses.

**Cytoplasmic DNA and RNA synthesis.** Cytoplasmic DNA synthesis was measured by following the uptake of 3H-thymidine into trichloroacetic acid-precipitable material in the cytoplasmic fractions. Cells were infected simultaneously with vaccinia virus and mengovirus. At selected times after infection, 10 μCi of 3H-thymidine were added to 10-ml samples of cells (5 × 10^6 cells/ml). After 20 min, the pulse was stopped by the addition of 10 ml of cold medium and cells were collected by centrifugation. They were then washed three times with cold tris(hydroxymethyl)-aminomethane (Tris)-buffered saline (TBS) and resuspended in 2.0 ml of 0.01 M phosphate buffer, pH 7.4. After allowing the cells to swell in this hypotonic medium for 10 min, they were homogenized with a Dounce homogenizer. Homogenization was monitored microscopically. It was found that under these condi-

<table>
<thead>
<tr>
<th>Table 1. Plaque assay with a single virus or a mixed pool of vaccinia and mengovirus</th>
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</thead>
<tbody>
<tr>
<td>Sample</td>
</tr>
<tr>
<td>Single virus</td>
</tr>
<tr>
<td>Mixture of viruses</td>
</tr>
</tbody>
</table>

a CEF, chicken embryo fibroblasts; PFU, plaque-forming units. 
b Mengo and vaccinia virus stocks were mixed and reassayed on the appropriate cells.
Disc-gel electrophoresis. Disc-gel electrophoresis of uninfected and infected extracts was performed by the technique of Holland and Kiehn (10) with several modifications. Electrophoresis was performed in 5% acrylamide gels. The gels were polymerized in 0.1 M Tris-acetate buffer (pH 9.0), 0.5 mM urea, 0.1% SDS, and 0.001% EDTA. The electrophoresis buffer consisted of 0.1 M Tris-acetate (pH 9.0), 0.05 M sodium acetate, 0.1% SDS, 0.01% EDTA, and 0.1% mercaptoethanol.

Mixed samples were run in columns 21-cm high and 6 mm in diameter. Electrophoresis was performed for 5 hr at 5 mA/column, constant amperage. The gels were pulverized in a Maizel fractionator (25) and, after drying, 0.1 ml of 0.1 N NaOH was added to each fraction, followed by 0.7 ml of NCS (Nuclear Chicago Solubilizer). After 2 hr, toluene-based scintillator was added, and the samples were counted in a Packard scintillation counter, set to measure $^{13}$C and $^{1}$H simultaneously. Under those conditions, recovery of counts per minute from the columns was 85 to 95%.

Materials used. Eagle's medium was purchased from the Grand Island Biological Co., Grand Island, N.Y., in the form of Joklik's modification of minimum essential medium. Radioisotopes were purchased from New England Nuclear Corp., Boston, Mass.; these were L-phenylalanine-$^{14}$C, 375.3 mCi/mmmole; L-valine-$^{14}$C, 219 mCi/mmmole; L-leucine-$^{14}$C, 240 mCi/mmmole; L-phenylalanine-$^{3}$H, 63 Ci/mmmole; L-valine-$^{2}$H, 2.34 Ci/mmmole; L-leucine-$^{4,5}$-$^{3}$H (N), 58.2 Ci/mmmole; uridine-$^{3}$H, 16.2 Ci/mmmole; thymidine-methyl-$^{3}$H, 10.6 Ci/mmmole. Acrylamide and N,N'-bis methylene acrylamide were purchased from Eastman Organic Chemicals, Rochester, N.Y.

RESULTS

Virus production in cells infected with both vaccinia virus and mengovirus. It can be seen (Table 2) that the yield of vaccinia virus after double infection was 1.2 to 3% of that obtained by infection with vaccinia virus alone. The results were the same whether cells were infected with both viruses at the same time or infected with mengovirus 1 or 5 hr after vaccinia virus infection. Mengovirus production was also restricted under these conditions. Other experiments in which the multiplicity of infection with mengovirus was reduced to 1 PFU/cell gave similar results.

Since the time required for maximum mengovirus production is 12 hr, as opposed to 22 to 24 hr for vaccinia virus production, it was possible that the reduction in the yield of vaccinia virus might be a reflection of the more rapid growth and killing of the cell by mengovirus, rather than an event related to the specific cessation of macromolecular synthesis resulting from mengovirus superinfection. To test these possibilities, cells were infected with vaccinia virus, and at various times a sample was removed and superinfected with mengovirus. After 1 hr of absorption of mengovirus, samples were chilled, washed three times with medium, and resuspended in the original volume of medium warmed to 37 C to continue the infection. A sample was taken at this point for virus assay, and the double infection was allowed to continue until 36 hr after the initial infection with vaccinia virus. The results of such an experiment are shown in Fig. 1. Washing the samples after mengovirus superinfection did not reduce vaccinia virus titer appreciably since, at early times, all virus is intracellular. A slight reduction is observed late in infection. Furthermore, an increase in vaccinia virus titer is observed at earlier times during absorption of mengovirus, probably pointing to the continuous replication of vaccinia virus during mengovirus absorption. The maximum amount of vaccinia virus produced after mengovirus superinfection was approximately equal to that produced during the first hour of superinfection with virus. This would indicate that inhibition of vaccinia virus replication occurs within 1 hr after mengovirus superinfection.

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Vaccinia virus titer</th>
<th>Mengovirus titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PFU/ml</td>
<td>Per cent of control</td>
</tr>
<tr>
<td>V alone</td>
<td>1.6 x 10⁷</td>
<td>100</td>
</tr>
<tr>
<td>V + M</td>
<td>2.0 x 10⁷</td>
<td>1.2</td>
</tr>
<tr>
<td>V + M (1 hr later)</td>
<td>2.0 x 10⁷</td>
<td>1.2</td>
</tr>
<tr>
<td>V + M (5 hr later)</td>
<td>5.0 x 10⁷</td>
<td>3.1</td>
</tr>
</tbody>
</table>

* L cells were infected with vaccinia virus [multiplicity: 5 plaque-forming units (PFU)/cell] and superinfected with mengovirus (multiplicity: 100 PFU/cell) at designated times after the initial vaccinia virus infection. Samples were assayed for virus yields 3 hr after the original vaccinia infection.

* V, vaccinia virus; M, mengovirus.

Vol. 8, 1971 DOUBLE INFECTION SYSTEM 295
The yields of mengovirus in the same experiment are shown in Table 3. The yields of mengovirus were again considerably reduced. In addition, it may be seen that mengovirus yield decreased as superinfection with mengovirus was delayed. The RNA virus stopped the continuous production of vaccinia virus, but it could not switch the system to maximal production of mengovirus.

The effect of superinfection of mengovirus-infected cells with vaccinia virus was also examined. Cells were first infected with mengovirus (100 PFU/cell); 1.5 and 3 hr later, the cells were superinfected with vaccinia virus (5 PFU/cell). The results are shown in Table 4. Mengovirus yields were reduced, but not as much as vaccinia virus yields were after mengovirus superinfection (Table 2).

DNA synthesis in the cytoplasm of double-infected cells. To characterize further the system of double infection, a study of the synthesis of DNA in the cytoplasm (vaccinia DNA) was performed. Cytoplasmic DNA synthesis was monitored as described above. Figure 2 shows that cytoplasmic DNA synthesis was delayed 1 to 1.5 hr in double-infected cells, but there was no appreciable reduction in the rate at which cytoplasmic DNA was synthesized.

Another experiment was performed in which the superinfection with mengovirus was delayed until the onset of vaccinia DNA replication. No effect was observed in this particular case on the rate of the ongoing replication of vaccinia DNA.

**Table 3. Effect of delayed superinfection on final yields of mengovirus**

<table>
<thead>
<tr>
<th>Sample (b)</th>
<th>Mengovirus titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PFU/ml</td>
</tr>
<tr>
<td>M alone</td>
<td>(1.2 \times 10^9)</td>
</tr>
<tr>
<td>V + M (1 hr later)</td>
<td>(2 \times 10^9)</td>
</tr>
<tr>
<td>V + M (5 hr later)</td>
<td>(9 \times 10^7)</td>
</tr>
<tr>
<td>V + M (9 hr later)</td>
<td>(7 \times 10^7)</td>
</tr>
<tr>
<td>V + M (12 hr later)</td>
<td>(1.7 \times 10^7)</td>
</tr>
<tr>
<td>V + M (15 hr later)</td>
<td>(1.6 \times 10^7)</td>
</tr>
<tr>
<td>V + M (26 hr later)</td>
<td>(2 \times 10^4)</td>
</tr>
</tbody>
</table>

\(a\) Mengovirus yields were measured 36 hr after the initial infection with vaccinia virus.

\(b\) V, vaccinia virus [multiplicity: 5 plaque-forming units (PFU)/cell]; M, mengovirus [multiplicity: 100 PFU/cell].

**Table 4. Yields of vaccinia virus and mengovirus in single- and double-infected cells**

<table>
<thead>
<tr>
<th>Sample (b)</th>
<th>Vaccinia virus titer</th>
<th>Mengovirus titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PFU/ml</td>
<td>Per cent of control</td>
</tr>
<tr>
<td>M alone</td>
<td>(&lt;10^6)</td>
<td>(&lt;2)</td>
</tr>
<tr>
<td>M + V (1.5 hr later)</td>
<td>(&lt;10^6)</td>
<td>(&lt;2)</td>
</tr>
<tr>
<td>M + V (3 hr later)</td>
<td>(&lt;10^6)</td>
<td>(&lt;2)</td>
</tr>
<tr>
<td>V alone</td>
<td>(5 \times 10^6)</td>
<td>100</td>
</tr>
</tbody>
</table>

\(a\) Yields of vaccinia virus were measured 32 hr after first infection, and yields of mengovirus were measured at 22 hr after infection.

\(b\) V, vaccinia virus [multiplicity: 5 plaque-forming units (PFU)/cell]; M, mengovirus [multiplicity: 100 PFU/cell].
fected.

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mengovirus-infected; N, infected cells.

hibited and then observed is a inhibition of infection under conditions of trichloroacetic acid-precipitable RNA.

is

with vaccinia virus infected

curve.

biphasic mengovirus-infected cells

thymidine for RNA synthesized (Table 5). Determined

porated effect of vaccinia virus with either 30-

material this later of mengovirus period of viral infection. There 'H-uridine.

there is a sudden rise at approximately 3 hr after infection. The rate of rise tends to decline about 4.5 hr, and then a second increase occurs that peaks about 7 hr after infection. The same type of curve is observed with either 30- or 10-min pulses of 'H-uridine. There was a very slight increase in the amount of precipitable radioactivity found in the cytoplasm of double-infected cells 5 and 8 hr after infection. This corresponds to the period of maximum appearance of similar material in cells infected only with mengovirus. Cells infected with vaccinia virus alone did not exhibit this later rise in trichloroacetic acid-precipitable material in the cytoplasmic extracts. A major effect of superinfecting mengovirus-infected cells with vaccinia virus appears to be the limitation of mengovirus viral RNA synthesis.

In the experiments in which appearance of DNA and RNA in the cytoplasm were measured, trichloroacetic acid-precipitable counts incorporated into the nuclear fraction were also determined (Table 5). The appearance of newly synthesized DNA in the nuclei was observed in single-infected and simultaneously double-infected cells. At 1.5 hr after infection, mengovirus-infected cells showed an inhibition of 60% above controls of uninfected cells as shown previously by Buck et al. (2), whereas the inhibition of vaccinia virus-infected cells was hardly detectable (less than 5% inhibition). Double-infected cells followed approximately the mengovirus-infected pattern (67% inhibition). When synthesis of nuclear RNA was observed, mengovirus-infected cells showed an inhibition of 65% compared to uninfected controls 3.5 hr postinfection, whereas

Appearance of newly synthesized RNA in the cytoplasm of double-infected cells. Figure 3 shows that under conditions of double infection newly synthesized RNA appearing in the cytoplasm of mengovirus-infected cells was somewhat inhibited (compared to uninfected cells) after infection with the virus and later presents a biphasic curve. After the initial inhibition, there is a sudden rise at approximately 3 hr after infection. The rate of rise tends to decline about 4.5 hr, and then a second increase occurs that peaks about 7 hr after infection. The same type of curve is observed with either 30- or 10-min pulses of 'H-uridine. There was a very slight increase in the amount of precipitable radioactivity found in the cytoplasm of double-infected cells 5 and 8 hr after infection. This corresponds to the period of maximum appearance of similar material in cells infected only with mengovirus. Cells infected with vaccinia virus alone did not exhibit this later rise in trichloroacetic acid-precipitable material in the cytoplasmic extracts. A major effect of superinfecting mengovirus-infected cells with vaccinia virus appears to be the limitation of mengovirus viral RNA synthesis.

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Table 5. DNA and RNA synthesis in the nuclei of double-infected cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Nuclear DNA synthesis 1.5 hr postinfection (per cent of control)</th>
<th>Nuclear RNA synthesis 3.5 hr postinfection (per cent of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Vaccinia virus-infected</td>
<td>95</td>
<td>90</td>
</tr>
<tr>
<td>Mengovirus-infected</td>
<td>40</td>
<td>35</td>
</tr>
<tr>
<td>Double-infected</td>
<td>33</td>
<td>30</td>
</tr>
</tbody>
</table>

* Cells were either single- or double-infected. At the indicated times after infection, either 'H-thymidine or 'H-uridine (1 µCi/ml) was incorporated for 20 and 30 min, respectively. Controls (uninfected cells) were labeled for the same periods of time, respectively. After breaking the cells in a hypotonic medium, nuclei were separated by centrifugation and washed several times; trichloroacetic acid-precipitable counts were determined.
vaccinia virus-infected cells showed only a 10% inhibition. The double-infected cells showed an inhibition of 70% compared to uninfected controls. This particular pattern was observed, both with 10- and 30-min pulses.

An experiment was run in which the addition of mengovirus was delayed for 2.5 hr after infection. Nuclear synthesis of DNA was followed. At 2.5 hr, vaccinia virus showed a slight inhibition with respect to uninfected controls. Upon addition of mengovirus, the inhibitory effect on nuclear DNA synthesis was observed immediately and at the same rate as when mengovirus is added simultaneously with vaccinia virus.

Study of protein synthesis during double infection. To study the synthesis of proteins during double infection, a preliminary experiment was run in which the uptake of \(^{3}H\)-leucine was followed in single- and double-infected cells. Pulses with \(^{3}H\)-leucine (1 \(\mu\)Ci/ml) were performed for a duration of 15 min. Radioactivity in hot trichloroacetic acid-precipitable material was determined (Fig. 4). In all samples, there was a general inhibition of protein synthesis that took place more slowly in vaccinia virus-infected than in mengovirus-infected cells. The double-infected cells show an inhibition of incorporation of at least the same magnitude as that of mengovirus-infected cells, similar to the effect observed in nuclear DNA and RNA synthesis. The vaccinia virus-infected cells show two maxima of incorporation: one at 2.5 to 3 hr and the other at 5.5 to 6 hr. In the mengovirus-infected system, there is a peak of incorporation at 5.5 to 6 hr after infection that roughly corresponds to the onset of synthesis of viral RNA. The double-infected cells show only a slight increase in protein synthesis between 5.5 to 6 hr after infection.

To determine the quality of the proteins synthesized during the different periods in single- or double-infected cells, analysis of proteins by disc-gel electrophoresis was performed. At the time when synthesis of host macromolecules is severely inhibited by both viruses (Table 5 and Fig. 4), it was possible to make a comparison between samples isolated from infected and uninfected cells. Uninfected cells were labeled for 20 min with \(^{14}C\)-labeled amino acids, whereas double-infected cells were labeled with \(^{3}H\)-labeled amino acids between 3 hr and 3 hr and 20 min after

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**Fig. 4.** Protein synthesis in single- and double-infected cells. Cells were allowed to incorporate \(^{3}H\)-leucine for 15 min. Hot trichloroacetic acid-precipitable counts were determined. Symbols: ●, vaccinia virus-infected; ○, mengovirus-infected; △, double-infected, 5 PFU/cell vaccinia, 100 PFU/cell mengovirus; □, uninfected.

**Fig. 5.** Gel electrophoresis of proteins synthesized in double-infected and uninfected cells. Uninfected cells were labeled for 20 min. Infected cells were labeled from 3 hr to 3 hr and 20 min after infection. Symbols: ●, \(^{14}C\)-proteins from uninfected cells; ○, \(^{3}H\)-double injected proteins. Electrophoresis was performed for 5 hr at 5 mA/column, constant amperage. The anode is on the right. Migration is from left to right.
infection. In general, there was an inhibition of synthesis of host proteins of high molecular weight after infection in double-infected (Fig. 5), vaccinia virus-infected, or mengovirus-infected cells, whereas proteins of low molecular weight accumulate.

The electrophoretic profiles obtained from single-infected cells labeled between 3 hr and 3 hr and 20 min after infection are represented in Fig. 6. Vaccinia proteins were labeled with \(^3\)H-labeled amino acids; mengovirus proteins were labeled with \(^1\)C-labeled amino acids between 3 hr and 3 hr and 20 min after infection. Both patterns are very different and a typical vaccinia virus peak (labeled VH3) can be detected.

In a subsequent experiment, vaccinia virus-induced and mengovirus-induced proteins were labeled with \(^1\)C-labeled amino acids, whereas \(^3\)H-labeled amino acids were used for labeling the double-infected culture. The results are shown in Fig. 7. The pattern from double-infected cells resembles the vaccinia virus-infected system (Fig. 7a) but with a clear accumulation of extra material of very low molecular weight. There was a peak present in extracts from vaccinia virus- and double-infected cells (peak labeled VH3 in Fig. 6, VC3 in Fig. 7a, and DH3 in Fig. 7b) that is absent in the mengovirus-infected cells (Fig. 7b). This was also seen in Fig. 6 where it was possible to compare directly patterns from vaccinia virus- and mengovirus-infected cells (VH3 peak is absent in MC3 pattern). Similar electrophoretic patterns as the ones obtained at 3 hr were observed when the infected cells were labeled between 2 hr and 2 hr and 20 min after infection.

Results of a pulse performed between 5 hr, 30 min and 5 hr, 50 min are shown in Fig. 8. Double-infected cells were, at that time, apparently synthesizing mostly mengovirus protein since the patterns obtained were more typical of those seen from mengovirus-infected cells (Fig. 8b) than from vaccinia virus-infected cells (Fig. 8a).

Summarizing, early in infection (up to 3 hr after infection) the double-infected cells are synthesizing vaccinia virus-like proteins and possibly some proteins typical of mengovirus infection. At 5 hr after infection, whatever proteins were being synthesized were electrophoretically more typical mengovirus proteins, vaccinia-like proteins being greatly reduced.

**DISCUSSION**

The results described show clearly a double interference in cells infected with both vaccinia virus and mengovirus. The effect was more drastic on the production of vaccinia virus, since no detectable increase was observed in the number of infective vaccinia particles produced in cells infected with both viruses. On the other hand, the production of mengovirus was also inhibited, although not to the same extent. The yield of infectious virus equaled about 15 to 20% of that obtained from cells infected only with mengovirus. This result is reproducible, as can be seen from Tables 3 and 4. Dales and Silverberg (5), however, reported no reduction in the yield of mengovirus in a system of double infection with vaccinia. This discrepancy could best be explained by differences in either L-cell lines or virus stocks used, as our results were the same regardless of multiplicity of infection of vaccinia virus or mengovirus. In fact, we consistently used vaccinia virus at a multiplicity of infection of 5 PFU/cell and found significant interference with mengovirus production, whereas Dales and Silverberg
performed their experiments with cells infected with vaccinia virus at a multiplicity of infection of 10 PFU/cell.

The steps at which the production of virus particles was inhibited appears to be different for vaccinia virus and mengovirus. When cytoplasmic DNA synthesis was examined in cells infected with both viruses, it was observed that production of vaccinia DNA was delayed for about 2 hr, but it was eventually synthesized in amounts approximately equal to that seen in cells infected only with vaccinia virus. On the other hand, the production of mengovirus RNA was severely hindered in double-infected cells. There was an approximate correlation between infectious mengovirus production and RNA and protein synthesized in double-infected cells.

The interference of mengovirus superinfection on the production of vaccinia virus appears to be a late phenomenon since production of vaccinia DNA is quantitatively normal. Also, the electrophoretic patterns of "early" proteins synthesized during the first 3 hr in double-infected cells correspond very closely to vaccinia virus proteins seen during the first 2 to 3 hr in cells infected only with vaccinia virus. However, at later periods of double infection, mainly protein peaks characteristic of mengovirus proteins were detected and characteristic "late" vaccinia proteins were absent (cf. Fig. 7 and 8).

The detection of vaccinia virus-like proteins by disc-gel electrophoresis and the synthesis of DNA in the cytoplasm of double-infected cells suggest that at least some vaccinia virus messenger RNA is synthesized and translated in cells superinfected with mengovirus. Interference of vaccinia virus replication by mengovirus, therefore, would appear to differ from interference caused by either exposure of cells to interferon (13) or the presence of replicating adenovirus (7). In these instances, vaccinia virus DNA and proteins were not synthesized because the early vaccinia virus messenger RNA failed to interact with host ribosomes. In the case of rifampin interference with vaccinia virus replication, the block appears to be at a step in viral maturation since most viral
proteins are synthesized but not assembled (16). Disc-gel comparisons of single- or double-infected cells would indicate that superinfection with mengovirus appeared to interfere with the synthesis of certain vaccinia virus proteins. Electron micrographs of double-infected cells did not reveal the presence of the viroplasm surrounded by a discontinuous membrane clearly seen in vaccinia virus-infected cells treated with rifampin (20; unpublished data). Therefore, the interference with vaccinia virus replication by superinfection with mengovirus also differs from the interference observed with rifampin.

Several hypotheses can be proposed for this particular kind of interference; however, interference at the level of virus adsorption can be ruled out. Simultaneous infection of cells with vaccinia virus and mengovirus did not inhibit the adsorption of vaccinia virus to the cells since the same inhibition effects were observed when superinfection with mengovirus was delayed as much as 5 hr. In simultaneous infection, there was a delay in the appearance of vaccinia virus DNA, so it is possible that the uncoating process was retarded by the presence of mengovirus. The vaccinia virus DNA-dependent RNA polymerase is carried by the virus (15), so little effect on the function of this particular protein might be expected. The delay in the appearance of vaccinia virus DNA could be due to a partial reduction in the rate of virus-directed protein synthesis since complete uncoating is necessary for the replication of DNA (19). Partially uncoated vaccinia virus particles, nevertheless, can be templates for the vaccinia virus DNA-dependent RNA polymerase (14). In HeLa cells, vaccinia virus early messenger RNA has a half-life of 120 min, whereas late messenger RNA has a half-life of only 13 min (24). In L cells, nevertheless, the stability of both kinds of messenger RNA is

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**Fig. 8.** Disc-gel electrophoresis of proteins synthesized in single- and double-infected cells from 5 hr, 30 min to 5 hr, 50 min after infection. (a) $^{14}$C-vaccinia virus proteins, ●; $^{3}$H-double-infected proteins, ○; (b) $^{14}$C-mengovirus proteins, ●; $^{3}$H-double-infected proteins, ○. Electrophoretic conditions as in Fig. 5.
approximately the same, but the amounts are very different (21). Late messenger RNA is hardly detectable. One might therefore expect a direct competition of vaccinia virus and mengovirus messengers for active ribosomes, and, as a result, late vaccinia virus RNA might not be read. A similar suggestion was made by Cords and Holland (4) to explain interference between enteroviruses. Mengovirus might also interfere with the synthesis of late vaccinia virus messenger RNA; once early messenger and early proteins were apparently synthesized. It may be, however, that each virus produces a "translation inhibitory protein" similar to that described in bacterial cells infected with T-even phages (8, 9, 11, 17, 26). If such was the case, the ribosomes of the cell would eventually be unable to synthesize effectively either host, mengovirus or vaccinia virus proteins, and the result would be the double interference reported here.

ACKNOWLEDGMENT

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LITERATURE CITED