# THE EFFECT OF MENGOVIRUS INFECTION ON THE ACTIVITY OF THE DNA-DEPENDENT RNA POLYMERASE OF L-CELLS. II. PRELIMINARY DATA ON THE INHIBITORY FACTOR.

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RNA synthesis is rapidly inhibited after infection of L-cells with Mengovirus and other viruses of the Columbia SK group (Martin et al., 1961; Baltimore and Franklin, 1962). This is due to the inhibition of the enzyme system responsible for synthesis of all species of RNA in the uninfected cell (Baltimore and Franklin, 1961). Recent evidence has indicated that such inhibitory activity requires the synthesis of protein during the early stages of infection (Franklin and Baltimore, 1962; 1963; Baltimore, Franklin, and Callender, 1963). This communication presents preliminary data on the characterization of the inhibitory factor. In order to measure inhibitory activity, nuclei of uninfected (control) cells were incubated with fractions from infected cells and then tested for ability to synthesize RNA.

## METHODS

L-cells were grown in a modified Eagle's spinner medium (Eagle, 1955) with 10% newborn calf serum. Cells were infected with Mengovirus at a multiplicity of 5-10. Details of infection and characteristics of virus growth have been described (Franklin, 1962; Baltimore and Franklin, 1962).

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For the present experiments cells were harvested three hours after infection, washed twice with .25M sucrose + $10^{-3}$M $Mg^{++}$, and then homogenized with a Dounce homogenizor in 10 volumes of demineralized water containing 0.005 M mercaptoethanol. Nuclei were prepared by centrifugation at 600g for 8' as described previously, and these nuclear pellets were used to assay the DNA-dependent RNA polymerase (Baltimore and Franklin, 1962). In the present experiments, nuclear and cytoplasmic fractions were present together in the assay tube and it was necessary to add an ATP-generating system (phospho(enol) pyruvate plus pyruvate kinase) to obtain maximal enzymatic activity. Details of the assay mixtures and procedures are presented in the tables.

## RESULTS

In Table I are presented data on DNA-dependent RNA polymerase activity in various mixtures of nuclei and cytoplasm from control or infected cells. As was previously reported, the inhibition of DNA-dependent RNA polymerase in infected nuclei is not dependent on the presence of infected cytoplasm (Baltimore and Franklin, 1962 and Table I,a). In the experiment of Table I the inhibition is 72.2% (b). In other experiments it was up to 95% complete at 3-4 hours after infection. Whereas incubation of control nuclei with cytoplasm from infected cells results in a 61% inhibition of control activity (c), mixing control nuclei and infected nuclei had no effect on the activity of the control nuclei (e). Mixing control cytoplasm with infected nuclei does not restore the activity of the infected nuclei (d). Thus the experiment presented in Table I shows that infected cytoplasm inhibits the activity of control nuclei and therefore suggests the existence of a cytoplasmic inhibitory factor.

The cytoplasmic inhibitory factor is inactivated by trypsin, as shown in Table II. Increasing concentrations of trypsin resulted in decreasing inhibitory activity. At a trypsin concentration of 25μg/ml, the inhibitory activity was completely destroyed. In other experiments the cytoplasm from infected cells was also incubated in the presence of Ribonuclease A

TABLE I. Inhibition of "aggregate enzyme" by a factor from infected cells.

| Nuclear preparation | mg.protein in 0.05 ml of nuclear preparation | Addition | mg.protein in 0.05 ml of addition | µµmoles ATP-8-$C^{14}$ incorporated/mg. nuclear protein |
|---|---|---|---|---|
| (a) Control | 1.35 | Control cytoplasm | 1.50 | 793 |
| (b) Infected | 1.15 | Infected cytoplasm | 1.35 | 220 |
| (c) Control | 1.35 | Infected cytoplasm | 1.35 | 309 |
| (d) Infected | 1.15 | Control cytoplasm | 1.50 | 161 |
| (e) Infected | 1.15 | Control nuclei | 1.35 | 806* |

Nuclei were prepared from control and infected cells and the supernatant of the 600g-8' centrifugation was considered to be the cytoplasm. In a total volume of 0.5 ml, the complete reaction mixture contained the following constituents; 50 µmoles Tris, pH 7.9 (37°), 1.5 µmoles $MnCl_2$, 10 µmoles NaF, 2.5 µmoles mercaptoethanol, 0.05 ml of saturated (room temperature) $(NH_4)_2 SO_4$, 0.2 µC ATP-8-$C^{14}$ (2.04 mC/mM), 60 µgm each of GTP, UTP, and CTP, 20 µg pyruvate kinase, 5 µmoles phospho(enol) pyruvate, 0.05 ml. nuclear preparation, and 0.05 ml. of the added cytoplasmic or nuclear fraction. The reaction mixture was incubated at 37°C for 60 minutes. The tubes were then chilled, 0.5 ml. of 0.1 M sodium pyrophosphate and 0.1 ml. of 0.1% bovine serum albumin were added, followed by 0.1 ml. of 10 N perchloric acid (PCA). After mixing, 4 ml. of 1 N PCA was added. The precipitates were washed three times with 4 ml. aliquots of 1 N PCA and the final pellets were dissolved in 0.5 ml. of concentrated formic acid and then transferred to planchets and dried. Radioactivity was measured with a Tracerlab-Omni-guard low background counter, having an average background of 0.8-1.0 cpm and a 20.3% efficiency for $C^{14}$.

*Calculated only on the basis of the control nuclei (1.35 mg protein/0.05 ml.).

TABLE II. Effect of trypsin on the inhibitory factor.

| Cytoplasmic Addition | Trypsin Concentration (µg/ml) | µµM ATP-8-$C^{14}$ incorporated/mg. nuclear protein | |
|---|---|---|---|
| | | Preincubated at 37°C | Preincubated at 0°C |
| (a) Control | 0 | 518 | 527 |
| (b) Infected | 0 | 349 | 368 |
| (c) Infected | 1 | 396 | 355 |
| (d) Infected | 5 | 427 | 381 |
| (e) Infected | 10 | 440 | 283 |
| (f) Infected | 25 | 558 | 405 |
| (g) Infected | 0 | 327 | 353 |

One 0.8 ml aliquot of control cytoplasm (a) and six 0.8 ml aliquots of infected cytoplasm (b-g) were mixed with the appropriate concentration of trypsin (Worthington, 2x crystallized) or 0.25 M sucrose + $10^{-3}$M Mg++ to give the final concentrations of trypsin indicated in the second column. Each of these aliquots was then divided into two 0.45 ml aliquots, one of which was incubated at 0°C and the other of which was incubated at 37°C, both for 30'. After this incubation, 0.05 ml (150 µg) of soybean trypsin inhibitor (Mann Research Lab., 5x crystallized), was added to each tube of series a and b. From these 14 aliquots (7 incubated at 0°C and 7 incubated at 37°C), 0.1 ml was mixed with 0.1 ml of control nuclei and this was assayed in a final volume of 0.5 ml as described in Table I.

(Worthington). After 30 minutes at 37°C the RNAase was inhibited by sulfonated polyvinylalcohol (Bernfield et al., 1960) and when this treated cytoplasm was tested with control nuclei, the inhibitor was still active.

The intracellular localization of the inhibitor was studied using fractional centrifugation (Table III). Although the "mitochondrial" fraction was very active, the "post-mitochondrial" (15,000 g, 10 min.) supernatant had the highest activity per mg. protein. The relative total amount of inhibitor per fraction was calculated as: (% inhibition ÷ 100) x total volume of fraction (last column of Table III). This clearly shows that the inhibitor is preponderantly localized in the "post-mitochondrial" supernant.

TABLE III. Localization of the inhibitory factor.

| Addition | mg.protein in 0.1 ml of addition | μμmoles ATP-8-$C^{14}$ incorporated/mg. nuclear protein | % inhibition | Relative total amt. of inhibitor per fraction |
|---|---|---|---|---|
| (a) total cytoplasm (supernatant of 600 g, 8') | 3.0 | 473 | 29.9 | 3.0 |
| (b) "mitochondrial" fraction (pellet of 5000 g, 10') | 6.8 | 396 | 41.3 | 0.4 |
| (c) "post-mitochondrial" fraction (pellet of 15,000 g, 10') | 4.9 | 616 | 8.6 | 0.1 |
| (d) supernatant of 15,000 g, 10' | 1.8 | 439 | 35 | 3.2 |
| (e) total cytoplasm of uninfected cell | 2.6 | 675 | 0 | - - |

The cytoplasmic fraction of the infected cell was further fractionated by centrifugation at 0°C in the Servall centrifuge with a SS-34 rotor. The sediments were taken up in 0.25 M sucrose + $10^{-3}$ M Mg++ and 0.1 ml aliquots were used in the complete reaction mixture (see Table I) with 0.1 ml. of nuclear enzyme preparation from uninfected cells. The reaction mixture was incubated at 37°C for 60 minutes.

## DISCUSSION

The present data are consistent with our hypothetical scheme of Mengovirus multiplication (Franklin and Baltimore, 1962; 1963). According to this scheme, viral RNA interacts with cellular ribosomes to form a viral protein synthesizing system in the cytoplasm. One of the viral specific proteins made early in infection may be the inhibitor discussed in this paper. The present data suggest that the inhibitor is made in the cytoplasm and then may be transported to the nucleus where it interacts with and inhibits the enzyme system (DNA template plus DNA-dependent RNA polymerase) responsible for normal RNA synthesis. In view of the recent demonstration of the inhibition of this RNA synthesizing system by histones (Huang and Bonner, 1962; Bonner and Huang, 1963), it is not unreasonable to propose that this viral specific protein could be a histone.

Our present studies are now directed towards an isolation and further characterization of this inhibitory factor, and to a further study of its mode of action.

## SUMMARY

A cytoplasmic factor in cells infected with Mengovirus is capable of inhibiting the enzyme system responsible for RNA synthesis in uninfected cells. This factor is sensitive to trypsin but not to ribonuclease and is found predominantly in the post-mitochondrial (15,000 g - 10') supernatant fraction.

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