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Mengovirus-Induced Cytopathic Effect in L-Cells: Protective Effect of Interferon

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The effect of interferon on mengovirus-induced cytopathic effect (CPE) in L cells, the cut-off of host-cell protein synthesis, and production of mature virus were found to be dependent on the concentration of interferon. CPE and inhibition of host protein synthesis were not affected until the concentration of interferon was increased 100-fold over that required to reduce viral yields by 90%.

Interferon at a concentration which reduces yields of mengovirus by 85 to 90% in L cells fails to prevent cell degeneration in 100% of the cells at the usual time (9, 12). This finding is surprising for the following reasons. The cytopathic effect (CPE) of mengovirus requires virus-directed synthesis of a cytopathic protein(s) [CPP, (5)]. However, since interferon is thought to act by altering host ribosomes to discriminate selectively against translation of viral messenger ribonucleic acid (mRNA) (11, 13-15), one would expect that synthesis of a CPP by mengovirus RNA would be inhibited by interferon. To explain this apparent inconsistency, one could suppose that either (i) early viral mRNA coding for CPP is much less sensitive to the action of interferon than later messages which result in mature virus, or (ii) the low level of translation of viral mRNA even in the presence of moderate concentrations of interferon results in accumulation of sufficient CPP to eventuate in cell death. In either case, if enough interferon were used it should be possible to reduce mengovirus-induced CPE in L cells. The present paper provides data in support of this concept.

MATERIALS AND METHODS

Cells. The CCL1 strain of mouse L cells was used throughout. The cells were maintained in spinner culture in BME supplemented with 10% fetal calf serum (FCS), glutamine and antibiotics (growth medium, GM). Monolayers were grown in four dram vials (Opticlear shell vials, 25 by 52 mm; Owen-Illinois Consumer & Technical Products Div., Toledo, Ohio) containing half of an 11- by 22-mm cover slip. Seeding vials with 2×10^5 cells in 1 ml of GM resulted in nearly confluent monolayers of 3.3×10^5 to 4×10^5 cells the following day. All experiments were carried out in a humid atmosphere with 5% CO_2 in air at 37 C.

Virus and virus assay. One mengovirus pool with a titer of 2×10^8 plaque-forming units (PFU)/ml was used in all experiments. Virus induced CPE and viral growth cycles were studied by infecting monolayers with 0.5 ml of mengovirus at a virus to cell input multiplicity of 20 to 1. After adsorption for 1 hr at 37 C, the monolayers were washed three times with BME to remove unadsorbed virus; the cultures were then incubated at 37 C with 1 ml of BME supplemented with 2% FCS, glutamine, and antibiotics (maintenance media, MM). To determine viral growth curves, cells were scraped at hourly intervals from duplicate vials with a rubber policeman and the cell suspensions were subjected to three cycles of freezing and thawing in a CO_2-alcohol ice bath. Infectivity was assayed by inoculating duplicate L-cell monolayers with 0.2 ml of serial dilutions of the lysates in MM. After adsorption at 37 C for 1 hr, the inoculum was discarded and 1 ml of an agar overlay consisting of 0.9% agar-Noble in Eagles tryptose phosphate base with 5% FCS, antibiotics, and 200 μg of diethylaminoethyl (DEAE)-dextran per ml (17) was added. After 24 hr of additional incubation, 0.5 ml of the agar overlay without DEAE-dextran but with 0.01% neutral red was added; the plaques were counted 12 hr later.

Interferon. Interferon was obtained in mouse serum after Newcastle disease virus infection (6) and assayed as previously described (7). The NIH reference standard is 500 units/ml by this assay procedure. In L cells, the titers are equivalent whether expressed in 0.5 log yield-reducing units or 50% plaque-reducing units. A single lot of pooled mouse sera with 100,000 0.5 log yield-reducing units/ml was appropriately diluted in MM in each experiment and incubated overnight with L cells. Comparable dilutions of a pool of normal mouse sera were added to control cultures.

Assay of cytopathic effect. Mengovirus-induced CPE in L cells was quantitated both by morphological methods and by determination of lysozomal activation. The latter has been shown to correlate with virus-induced CPE (1). Lysozomal activity was assayed histochemically by a modified Gomorri technique (1). Cells that exhibited staining for acid phosphatase in discrete bodies (Fig. 1A) or extensive

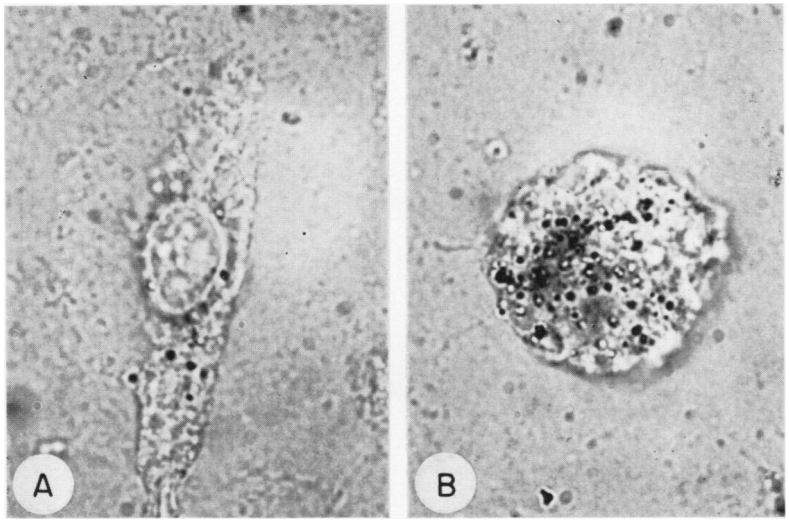

FIG. 1. *Histochemical staining of acid phosphatase in lysosomes. (A) First-stage lysosomal activation; (B) second-stage lysosomal activation. Photomicrograph. × 1,000.*

staining extending into the cytoplasm (Fig. 1B) were considered to be damaged. These changes in staining reflect increased permeability of the lysozomal membrane to the enzyme substrate, β-glycerol phosphate, and are referred to as first and second stage lysozomal activation, respectively.

CPE was quantitated by combining the results of the lysozomal assay with those obtained by examination of the cells by ordinary light and phase microscopy. Cells showing extensive vacuolization, rounding, and nuclear pycnosis or staining of lysozomes, or both, were considered damaged. In experiments performed in triplicate, 200 cells per cover slip were examined and the percentage of damaged cells was recorded and averaged.

Incorporation of radioactive metabolites. ^{14}C-valine (0.2 c/mmole) was purchased from New England Nuclear Corp., Boston, Mass. Spinner cultures infected at a multiplicity of infection of 20 to 1 were diluted in valine-free BME and labeled at intervals with 1 μc per 15-ml sample. Incorporation of the radioactive metabolite into the trichloroacetic acid-insoluble precipitate was taken as a measure of protein synthesis. Counting was done in a Packard Tri-Carb scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.) on acid-insoluble precipitates collected on membrane filters (Schleicher & Schuell Co., Keene, N.H).

RESULTS

Effect of interferon concentration on mengovirus-induced CPE in L cells. Interferon at concentrations which markedly reduce viral yields, 10 and 100 units/ml, did not affect CPE in infected L cells as previously noted (9, 12). Higher concentrations of 1,000 and 10,000 units/ml, however, effected a delay in the time of appearance of CPE and slowed the rate of development of CPE (Fig. 2). Cells treated with these higher concentrations of interferon appeared morphologically normal 10 to 12 hr postinfection when more than 75% of infected cells not exposed to interferon exhibited advanced cellular degeneration (Fig. 3A and B). It is possible that CPE might be completely suppressed by interferon at concentrations in excess of 10,000 units/ml since nearly 50% of the cells treated with 10,000 units of interferon did not exhibit CPE 24 hr postinfection.

Effect of increased concentrations of interferon on the cut-off of cell protein synthesis. Within 2

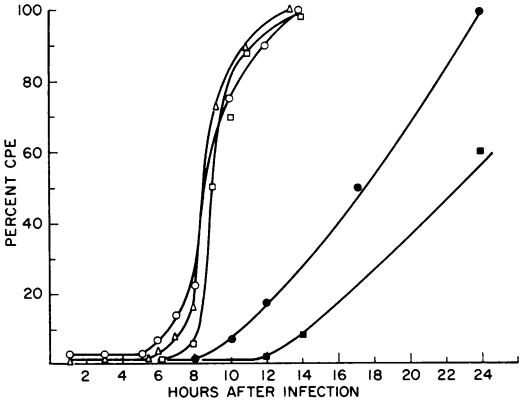

FIG. 2. *Effect of interferon concentration on mengovirus induced CPE. Symbols: ○, infected cells; △, infected cells pretreated with 10 units interferon; □, infected cells pretreated with 100 units of interferon; ●, infected cells pretreated with 1,000 units of interferon; ■, infected cells pretreated with 10,000 units of interferon.*

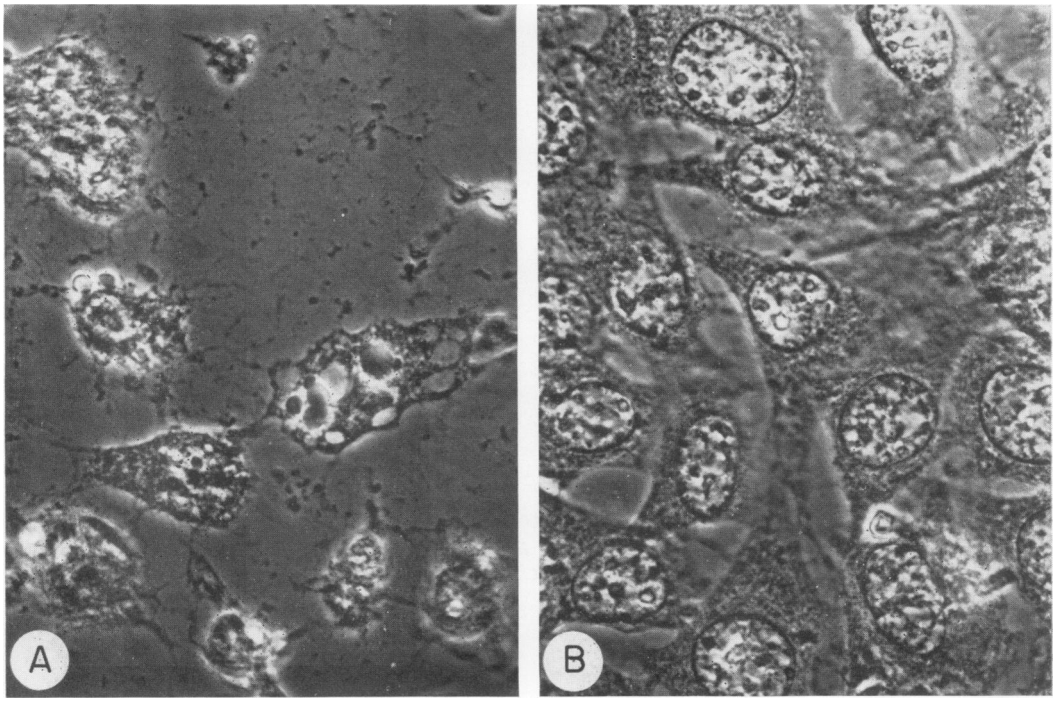

FIG. 3. *Effect of 1,000 units of interferon on mengovirus-induced CPE. (A) CPE in L cells 10 hr after infection with mengovirus; (B) L cells pretreated with 1,000 units of interferon 10 hr postinfection. Phase microscopy. × 1,000.*

hr postinfection, proteins synthesized under control of mengovirus RNA inhibit synthesis of cell RNA and protein (5). It had been shown previously that interferon at a concentration which did not prevent CPE would delay the cut-off of RNA synthesis by 1 hr, but did not affect the mengovirus-induced inhibition of protein synthesis (12). Since interferon did diminish CPE at high concentrations, it was of interest to determine if increasing the concentration of interferon would also affect the cut-off of protein synthesis. Figure 4 depicts the effect of 1,000 units of interferon on the rates of protein synthesis at intervals following infection of spinner cultures of L cells. The rapid cut-off of protein synthesis in infected cells was delayed by 3 hr by exposure to interferon at the increased concentration. The rise in protein synthesis during virus production was also less, presumably reflecting the decrease in virus yields in the interferon-treated cells. By 6 hr postinfection, comparable reductions of protein synthesis were evident in infected cells with or without exposure to interferon.

The relationship of the cytopathic protein to inhibition of macromolecular synthesis. The experiments to be described had two aims: (i) to confirm the dependence of CPE on virus-directed protein synthesis and (ii) to show that the proteins responsible for inhibition of host cell protein and RNA synthesis are different from the protein(s) responsible for CPE.

In accord with the observations of others (2, 9), we found that mengovirus-induced CPE requires virus-directed protein synthesis and that the CPP accumulates in cells at the end of the viral latency period. The requirement for, and time of synthesis of, the CPP was determined by replacing media of infected cultures at 0.5-hr intervals postinfection with media containing 25 μg/ml of cycloheximide, a compound that rapidly blocks protein synthesis by inhibiting the transfer of amino acids from aminoacyl-soluble RNA to growing polypeptide chains on the ribosome (8). Additions of cycloheximide up to 4 hr postinfection prevented the development of CPE; additions of cycloheximide after this time resulted in CPE indistinguishable in time of appearance and rate of development from infected monolayers not exposed to cycloheximide. Since new virus is first detected 5 hr postinfection (Fig. 6), the CPP must have accumulated in cells at the end of the viral latency period.

Two lines of evidence suggest that the proteins responsible for depression of host-cell macro-

molecular synthesis are not the same as the CPP(s). First, the "cut-off" proteins are present and exert maximum effects by 2 hr postinfection (5), whereas CPP(s) appears 4 hr postinfection with maximum effects 8 hr postinfection. Further evidence that CPP and the cut-off proteins are different was obtained by studying the effect of inhibitors of protein and RNA synthesis on the development of CPE. Actinomycin D and puromycin, inhibitors of deoxyribonucleic acid (DNA)-dependent RNA synthesis and protein synthesis, respectively, were incubated with L-cell monolayers at concentration of 10 μg of actinomycin per ml and 200 μg of puromycin per ml that have been shown to inhibit 98 to 99% of RNA and protein synthesis in L cells (4, 10). The extent of CPE was then compared to that seen in infected cells at corresponding times postinfection. The dramatic CPE induced by viral infection was not observed in the cells incubated with the metabolic inhibitors, although by 24 hr of incubation up to 30% of the cells showed extensive vacuolization and rounding. Therefore, it appears that not only is the CPP synthesized later in infection than the cut-off proteins, but also the action of the cut-off proteins on L cells could not account for the effect of the CPP.

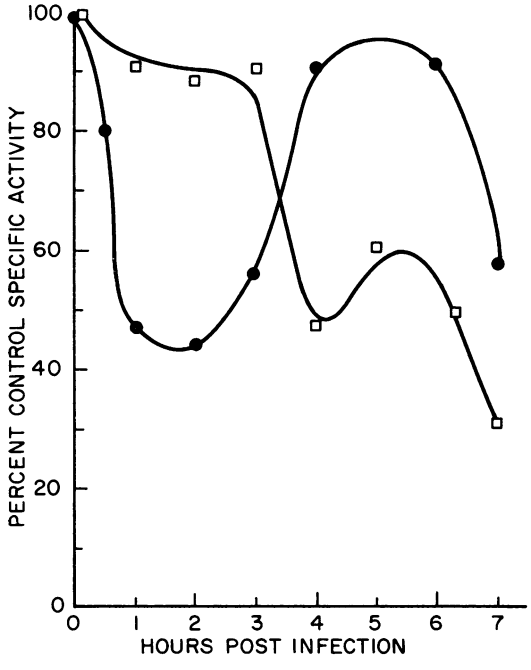

FIG. 4. *Effect of 1,000 units of interferon on protein synthesis in mengovirus-infected L cells. Incorporation of ^{14}C-valine into the trichloroacetic acid-insoluble precipitate of spinner cultures of L cells. Symbols: ●, infected cultures; □, infected cultures pretreated with 1,000 units of interferon. Per cent specific activity based on 520 counts per min/μg of protein incorporation in the control culture.*

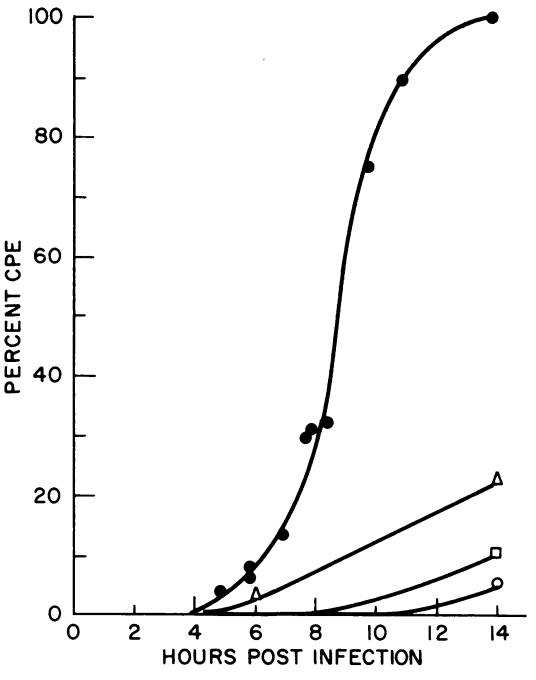

FIG. 5. *Inhibition of CPE by cycloheximide. Cycloheximide 25 μg/ml added to infected monolayers of cells. ○, 2 hr postinfection; □, 3 hr postinfection; △, 4 hr postinfection; ●, 5 hr postinfection.*

DISCUSSION

The data reported here indicate that two effects of mengovirus on L cells, the production of CPE and cut-off of host cell protein synthesis, are affected by interferon, but only at concentrations of interferon 100 times that required to reduce yields of mature virus by 90%. Two alternative explanations can be offered for the relative insensitivity of these two viral effects. One is that there are simply portions of the viral genome that are inherently resistant to the action of interferon. The second is that even though viral mRNA is translated with low efficiency on interferon-altered ribosomes, there is, nevertheless, sufficient synthesis of the cytopathic or cut-off proteins to have their full effect on the host cell. In contrast, the low efficiency in translation of viral mRNA at each of several dependent steps in the viral growth cycle would be cumulative and thereby block production of mature virus.

There are several reasons to attribute the effects of mouse-serum interferon on virus-induced CPE and inhibition of protein synthesis to interferon

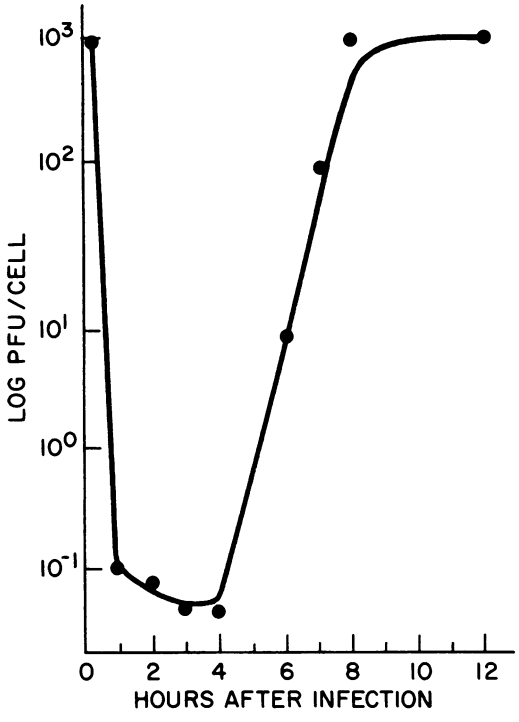

FIG. 6. *Growth curve of mengovirus in CCL1 subline of L cells.*

and not to increased concentrations of other factors in the serum. First, the anti-CPE had the following properties in common with interferon: species specificity (CPE was affected in mouse cells but not in chicken cells), lack of viral specificity (CPE induced by Semliki Forest virus and vesicular stomatitis virus was also inhibited), stability at *p*H 2, and cell-bound activity (not removed by washing prior to viral challenge). In addition, the mouse serum with interferon had been characterized fully for activity against viral replication and met criteria for interferon, namely, trypsin sensitivity, nonsedimentability, requirement for RNA and protein synthesis as well as the properties listed above. Finally, normal mouse serum treated identically and used at the same concentration on control cultures did not affect induction of CPE or inhibition of protein synthesis.

The relationship of mengovirus-induced CPE to virus-directed synthesis of a protein(s) that accumulates in cells at the end of the viral latency period was confirmed (2, 9). To support our contention that the concentration effect of interferon held for two viral events, induction of CPE and inhibition of protein synthesis, experiments were performed to demonstrate that the cut-off proteins were different from the CPP(s). That these proteins are different is suggested by the timing experiments with inhibitors of protein synthesis that show that the virus-induced cut-off proteins which depress host cell protein and RNA synthesis are present and have their effect in L cells at least 2 hr prior to synthesis of CPP. In addition, the inability of almost total inhibition of host protein and RNA synthesis by metabolic inhibitors to mimic viral CPE is further evidence that the CPP and the cut-off proteins are distinct and have different effects on the cells. The situation with another picornavirus, polio virus, is similar where it has been shown (4) that CPE does not occur in infected cells treated with guanidine, but nearly total depression of host-cell macromolecular synthesis does occur under these conditions. In the case of vaccinia virus, it also appears that the protein(s) responsible for inhibition of macromolecular synthesis in host cells and the protein which causes CPE are separate since the latter protein must be synthesized after viral infection (3), whereas the inhibitor proteins are already present on the intact viral particle (16).

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