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Destruction of L Cells by Mengo Virus: Use of Interferon to Study the Mechanism

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Interferon, when added to L cells, inhibited the synthesis of infectious Mengo viral ribonucleic acid, hemagglutinins, and infectious virus by 85 to 95%. Serum-blocking antigens were also reduced by the action of interferon, but threefold excess amounts of these antigens accumulated in interferon-treated cultures above the amounts expected for the quantity of infectious virus that was produced in these cultures. Radioautographic analysis showed that 28 to 36% of the cells of an interferon-treated population synthesized viral ribonucleic acid and 36 to 47% produced viral antigens as determined by an immunofluorescence technique. Despite the reductions in synthesis of viral components, all cells in an interferon-treated culture underwent cytopathic effects at the same time as cells in infected cultures which had not been treated with interferon. The results are compatible with the hypothesis that the cell destruction which results from the infection of L cells with Mengo virus is due to a protein which is coded for by the virus but is not a component of the mature virion.

The mechanism(s) by which cells are destroyed after infections by viruses is unknown. In the case of two small ribonucleic acid (RNA) viruses, polio (3) and Mengo (2), it has been hypothesized that capsid proteins are responsible for the resulting cell destruction. Both of these studies used inhibitors of protein synthesis to establish that protein synthesis is required at a time near the end of the latent period for manifestation of cytopathic effects. It was found that initiation of synthesis of viral coat proteins occurs simultaneously. Previous studies by Gauntt and Lockart (9) with the Mengo virus-L cell system were in agreement with those studies, on the time at which protein synthesis is required for subsequent cytotoxic effects. In addition, we showed that interferon inhibited the production of virus in 66 to 69% of the cells but failed to prevent complete destruction of the entire culture at the usual time (9). In the present study, interferon was used to block virus production in an attempt to determine whether the presence of viral capsid proteins is a prerequisite to cell destruction. The levels of accumulations of viral RNA and coat proteins in interferon-treated and control cultures were determined, and their distribution in individual cells was elucidated. The results are compatible with the hypothesis that the cytopathic effects resulting from Mengo virus infection of L cells are due to a protein(s) which is not a constituent of the mature virus.

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MATERIALS AND METHODS

Cells and virus. Methods for the growth and maintenance of a subline of L-929 cells (designated Lts) were previously described (9). Procedures for the production and assay of interferon were previously outlined (9). By this assay, one protective unit (PU) of interferon is designated as that concentration which prevents the appearance of cytopathic effects in Lts cell cultures (ca. 1.2 million cells) subsequent to challenge by 10 plaque-forming units (PFU) per cell of Western equine encephalomyelitis virus at 12 to 15 hr after interferon addition (13). Samples of high-titer stocks of interferon (500 to 1,250 PU/ml) were thawed from storage at −20 C and diluted in growth medium for each experiment.

Assay of virus and interferon. Procedures for the production and assay of Mengo virus have been described (9). Growth of this strain of Mengo virus was maximally inhibited by incubating cultures of Lts cells for 16 to 18 hr with 10 PU of interferon per ml prior to challenge with virus (9). This concentration of interferon (10 PU/ml) was used throughout. A 2-ml amount of interferon or growth medium was added to monolayer cultures grown in 60-mm petri dishes which contained 0.8 to 1.2 million cells (hemagglutinin and serum-blocking antigen experiments) or 0.6 to 0.8 million cells (radioautography and immunofluorescence tests). Monolayer cultures from which RNA

was to be extracted were grown in 100-mm petri dishes (4 to 6 million cells) and were incubated with 6 ml of interferon (5 PU/ml) or growth medium.

Hemagglutination test. Hemagglutination titrations of virus, using sheep erythrocytes, were performed on samples which had been subjected to three cycles of rapid freezing and thawing followed by centrifugation at 700 × *g* for 5 min. All dilutions were made in phosphate-buffered saline (6) containing 0.05% bovine albumin (PBSA) lacking antibiotics; 0.5 ml of each dilution was mixed with 0.5 ml of a 1% solution of sheep erythrocytes. The mixtures were incubated for 4 hr at 25 C. End points were expressed in hemagglutinin units, one unit being defined as the reciprocal of the final dilution which caused partial hemagglutination.

Serum-blocking power test for viral protein. The serum-blocking power test, used to compare the amounts of virion protein produced in control and interferon-treated cultures, was performed as follows. Samples were initially subjected to three cycles of rapid freezing and thawing, followed by centrifugation at 700 × *g* for 5 min. Portions of each test sample were then exposed to an 8-w General Electric germicidal lamp at a distance of 15 cm for 20 sec to reduce the number of PFU by approximately 100-fold. Portions (0.5 ml) of various dilutions of the ultraviolet-inactivated test samples were mixed with equal volumes of Mengo virus antiserum which had been standardized to neutralize 90% of a virus suspension containing 2×10^8 PFU. After the ultraviolet-inactivated virus-antiserum mixtures were incubated for 30 min at 37 C, 0.5 ml of a standard virus suspension containing 2×10^8 PFU was added to each mixture, and the mixtures were incubated an additional 30 min at 37 C. Each mixture was then immediately assayed by the plaque technique for the content of standardized virus not neutralized. Controls were run with each test to insure the neutralizing capacity of the antiserum. Titrations were performed on the ultraviolet-irradiated test samples to determine that at least 99% of the infectious virus had been inactivated. All dilutions were made in PBSA.

The antiserum used in the serum-blocking power test was obtained from rabbits which had been injected intraperitoneally every 2 days with virus lysate containing 2×10^9 PFU (per ml) of Mengo virus. Initial injections of 0.5 ml were increased up to 3.0 ml for the final injection. Ten injections were given over a 3-week period. The sera were collected in the usual manner and stored at 0 C without preservative. This antiserum had a k value of 31 min^{-1} (1).

Monolayer cultures were incubated with interferon or growth medium for 16 to 18 hr. The cell layers were then washed, and an inoculum containing 20 PFU per cell of Mengo virus and 10 μg of actinomycin D per ml was added to each. After a 1-hr period at room temperature for adsorption, the cultures were washed three times with PBSA lacking antibiotics. Uninfected cultures were similarly incubated with 10 μg of actinomycin D per ml during the adsorption period. Growth medium, lacking antibiotics and containing ^{3}H-uridine at 1 μc/ml, was added to each culture. All cultures were harvested at 9 hr postinoculation by scraping the cells into the medium; after centrifugation at 500 × *g* for 5 min, the supernatant fluid was removed. The fractions were stored separately at −10 C and recombined prior to extraction of nucleic acids.

Since the effect of interferon on the synthesis of Mengo virus-specific RNA was to be examined, it was necessary to add actinomycin D to infected cells to prevent the uptake of ^{3}H-uridine into cell ribosomal RNA (18). Actinomycin D is known to partially reverse the viral inhibition resulting from the action of interferon (13, 21), although it does not affect the synthesis of Mengo virus (8, 11, 18). Experiments were therefore performed to determine the length of time that cells had to be incubated with interferon to avoid reversing its inhibitory effect when actinomycin D was added. For this purpose, monolayer cultures of Lts cells were incubated with interferon or growth medium for various periods of time. Each culture was challenged with an inoculum of 20 PFU per cell of Mengo virus which contained actinomycin D at a concentration of 10 μg/ml. The yields of virus at 24 hr postinoculation were determined in two monolayer cultures. The results of a typical experiment are shown in Fig. 1. Inhibition of Mengo virus growth by interferon was partially reversed (13 to 27%) when actinomycin D was added to cultures previously incubated with interferon for less than 12 hr. There was little or no reversal of the inhibition if cultures had been treated for 16 to 18 hr with interferon before infection and addition of actinomycin D. Therefore, in all succeeding experiments, cultures were incubated with interferon for 16 to 18 hr prior to challenge with an inoculum containing Mengo virus and 10 μg of actinomycin D per ml.

Isolation and analysis of RNA. Nucleic acids were isolated from cells and virus by extraction with phenol and sodium dodecyl sulfate (19). The ethyl alcohol-precipitated nucleic acids were resuspended in STEP buffer (0.01 M sodium acetate buffer adjusted to *p*H 5.0, 0.005 M-tris(hydroxymethyl)aminomethane, *p*H 5.0, 0.04% sodium ethylenediaminetetraacetate, and 20 μg of polyvinyl sulfate per ml). The infectious RNA content of a preparation was assayed in Lts cell monolayers by the usual plaque technique (9), which was modified by the following additional steps. All dilutions of infectious RNA were made in PBSA lacking antibiotics and containing 500 μg of diethylaminoethyl-dextran per ml. All dilutions were kept at 0 C. Samples of each dilution were allowed to adsorb for 20 min at 25 C to monolayer cultures which had been washed twice with PBSA lacking antibiotics. The inoculum was redistributed at 10 min postinoculation. At the end of the adsorption period, 10 ml of the nutrient agar overlay was added to each culture (9). After incubation for 48 hr at 37 C, plaques were delineated by staining with 0.01% neutral red in PBSA. If titrations of infectious RNA were performed in the absence of diethylaminoethyl-dextran, no plaques resulted.

Radioautography. The cover slip-culture technique of Franklin (7) was used in the radioautography and immunofluorescence experiments. After a 1-hr period for virus adsorption at 25 C, the inoculum which contained 10 μg of actinomycin D per ml was removed.

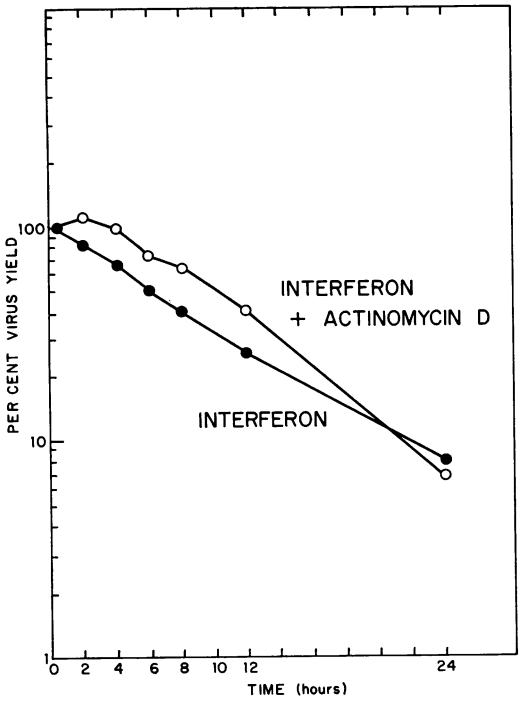

FIG. 1. *Reversal by actinomycin D of the interferon-mediated inhibition of Mengo virus replication. Duplicate monolayer cultures containing 4.0 million cells were incubated with interferon for various periods of time, washed with PBSA, and infected with 20 PFU per cell of Mengo virus containing 10 μg of actinomycin D per ml. After 1 hr of adsorption, free virus was removed by three washes with PBSA. Duplicate cultures were withdrawn 24 hr postinoculation, and the cells were subjected to three cycles of rapid freezing and thawing. The samples were frozen until assayed for virus. Time (hr) represents the number of hours that duplicate cultures were incubated with interferon prior to challenge by Mengo virus with* (○) *or without* (●) *actinomycin D in the inoculum. Virus production was 520 and 400 PFU per cell in control cultures not treated with actinomycin D and in cultures treated with actinomycin D, respectively.*

Control uninfected cultures were also incubated with this concentration of actinomycin D during the adsorption period. The following procedure was adopted for the radioautographic studies. At 4 or 9 hr postinoculation, 0.2 ml of warm growth medium containing 1 μc of ^{3}H-uridine was added to each culture. Control cultures not challenged with virus or treated with actinomycin D were included. After the 2-hr period of incubation with tritiated uridine, the radioactive medium was removed, and the cultures were fixed at 0 C for 10 min by addition of a modified Carnoy's solution (3:1, ethyl alcohol-glacial acetic acid) for 10 min at 0 C. Each cover slip was rinsed five times in a series of alcohol washes (90, 70, 40, 20, and 10%) and then in deionized water. The fixed cultures were treated twice with 5% cold trichloroacetic acid for 5 min at 0 C, washed exhaustively with deionized water, 95% ethyl alcohol, ethyl alcohol-ether (1:1), and ether, and air-dried. The cover slips were fastened to glass microscope slides with Permount and covered with Kodak AR-10 stripping film. The film was exposed for 5 days at 0 C and developed in Kodak D19. Cells were stained with hematoxylin. Photomicrographs were taken with a Leitz camera.

Immunofluorescence technique. The procedure followed in the indirect immunofluorescence test was as follows. After the infection of cover-slip cultures, as outlined above, the cover-slip cultures were fixed at 11 to 12 hr postinoculation with acetone at 0 C. The fixed cultures were stored at −70 C until the test was performed. The anti-Mengo serum used in this test was obtained from rabbits after an injection schedule similar to that described above. The rabbits were injected with a virus lysate containing virus which was grown in Chinese hamster ovary cells (23). (These cells were a kind gift of Robert Tobey of the Los Alamos Scientific Laboratory.) Nonspecific background fluorescence, as observed in uninfected control cells, was reduced by adsorbing the antiserum three times with mouse liver powders (Baltimore Biological Laboratories) at a concentration of 100 μg/ml. This antiserum had a k value of 27 min^{-1} and was used undiluted in the test (1). Fluorescence-conjugated anti-rabbit γ-globulin goat serum (Hyland Laboratories) was also cross-adsorbed three times with the mouse liver powders and used at a 1:10 dilution. The indirect test was performed by adding the anti-Mengo serum to the dry cover-slip cultures and allowing the reaction to proceed at 37 C for 30 min in a moist CO_2 incubator. The anti-Mengo serum was removed and the cover-slip cultures were washed five times with phosphate-buffered saline and then blotted dry. Conjugated anti-rabbit γ-globulin goat serum was then added to the cover-slip cultures, and they were incubated an additional 30 min under the same conditions. The cover-slip cultures were washed five times with distilled water, air-dried, and mounted on slides with Elvanol (polyvinyl alcohol, Dupont). Observations were made with a Zeiss fluorescence microscope employing a dark-field condenser. Nonspecific fluorescence was a dull green color, whereas immunofluorescence was bright yellow.

RESULTS

Effect of interferon on the yield of infectious Mengo RNA. Interferon has been shown to inhibit the synthesis of the RNA of several viruses (5, 10, 14). Two reports have shown quantitative relationships between the inhibition, by interferon, of yields of infectious Western equine encephalomyelitis virus (14) and Eastern equine encephalomyelitis virus (10) and their respective infectious viral RNA molecules. In the present work, the effect of interferon on the yields of Mengo virus and Mengo virus-infectious RNA was quantitatively determined. The purpose of these experiments was to ascertain whether infec-

tious RNA was synthesized in sufficient quantity to play a role in destroying nonvirus-producing cells (9). Monolayer cultures of Lts cells were incubated with interferon or with growth medium for 16 to 18 hr. Each culture was washed with PBSA lacking antibiotics and then challenged by the addition of 20 PFU per cell of Mengo virus in PBSA which contained 10 μg of actinomycin D per ml. Incubation at 37 C for 1 hr was allowed for the virus to adsorb. The cultures were then washed three times with PBSA lacking antibiotics, and medium was added. At 9 hr postinoculation, the cultures were harvested and the RNA was extracted and assayed for infectivity. Virus yields at 9 hr postinoculation were determined on other portions of each culture. The yields of infectious RNA and infectious virus from interferon-treated cultures are reported in Table 1 as percentages of the values found in control cultures without interferon. A close correlation between the levels of inhibition of yields of infectious virus and infectious RNA was found in interferon-treated cultures. The results establish that excess uncoated infectious RNA is not synthesized in interferon-treated cultures, and therefore infectious RNA is probably not involved in the destruction of nonvirus-producing cells.

Radioautographic study of ^{3}H-uridine incorporation into trichloroacetic-precipitable material in interferon-treated and control cultures. The preceding experiments established that interferon inhibited the synthesis of Mengo virus-infectious RNA. This did not rule out the synthesis of noninfectious viral RNA. To determine the proportion of cells which synthesized viral RNA, a radioautographic technique was used. Coverslip cultures of L cells were incubated with interferon or growth medium for 16 hr prior to challenge with 20 PFU per cell of Mengo virus. Actinomycin D at 10 μg/ml was added to the challenge inoculum and also to the phosphate-buffered saline in which the uninfected cultures were incubated during the adsorption periods. After adsorption of virus, the inoculum was removed and warm growth medium was added to each culture. At 5 hr postinoculation, warm growth medium containing tritiated uridine (1 μc/ml) was added to each culture, and the cultures were incubated for an additional 2 hr. Uninfected control cultures, not treated with actinomycin D, were included.

TABLE 1. *Effect of interferon on yields of infectious virus and infectious RNA*[a]

Expt no.	Virus yields[b] (% of controls)	Infectious RNA yields[c] (% of controls)
1	6.1	3.1
2	6.2	9.1
3	8.9	5.3

[a] Monolayer cultures which contained 9.3, 8.0, and 9.2 million cells in experiments 1, 2, and 3, respectively, were incubated for 16 to 18 hr with 10 PU per ml of interferon.

[b] Control cultures produced an average of 990, 462, and 630 PFU per cell at 9 hr postinoculation in experiments 1, 2, and 3, respectively.

[c] Control cultures yielded an average of 0.026, 0.011, and 0.19 infectious RNA plaques per cell at 9 hr postinoculation in experiments 1, 2, and 3, respectively.

Very few grains were found in the uninfected actinomycin D-treated cells (Fig. 2A). Grain counts of the cytoplasmic areas of these cells were made to establish a background count. Virus-infected cells not treated with interferon are shown in Fig. 2B; virus-infected cells which had been incubated with interferon are shown in Fig. 2C. A positive cell in the interferon-treated population was one in which the grain count was above the background count found in uninfected actinomycin D-treated cells. No attempt was made to count the number of grains in cells from either of the infected populations because of the inaccuracy of counting the very large number of grains found in most cells. If the cells were treated with interferon prior to infection in the presence of actinomycin D, a reduction in the number of positive cells was observed (Fig. 2C). Most of the cells scored as positive from this population did not exhibit as many grains as an average positive cell from the virus control population. A quantitative analysis of the number of positive cells in both cultures is shown in Table 2. In these experiments, 90 to 96% of the control cells synthesized viral RNA. When these control values were taken as 100% positive, it was found that 28 to 37% of the cells in an interferon-treated population synthesized viral RNA. These results correlate well with the previous findings (9) that 31 to 34% of cells in an interferon-treated culture produced infectious virus. The data suggest that synthesis of viral RNA is not involved in destruction of nonvirus-producing cells.

Effect of interferon on production of hemagglutinins. The levels of accumulation of two viral-coat proteins in interferon-treated and control cultures were determined by two different methods. Since some virus is produced in interferon-treated cultures, a ratio of the amounts of viral protein synthesized per plaque-forming unit produced in each culture would afford a means of detecting synthesis of excess proteins in interferon-treated cultures. Experiments to determine the amount of hemagglutinins synthesized in inter-

feron-treated cultures were performed. The hemagglutination titrations were performed on samples of cultures harvested at 16 hr postinoculation. The results from three experiments are shown in Table 3.

Clearly, interferon inhibited the synthesis of hemagglutinins, and approximately to the same extent that infectious virus yields were reduced. A comparison of the PFU per hemagglutinin unit ratio between interferon-treated and control cultures shows that no excess hemagglutinins were synthesized in interferon-treated infected cells.

Effect of interferon on the production of serum-blocking antigens. An additional method, the serum-blocking power test, was used to measure viral proteins—specifically, the accumulation of coat proteins. This test was performed on control and interferon-treated monolayer cultures harvested at 16 or 24 hr postinoculation. Samples were titered for their active virus contents. Portions of various dilutions of ultraviolet-inactivated test samples were tested for their content of serum-blocking antigens. The dilutions of the samples from the control and interferon-treated cultures which achieved similar degrees of reduction of neutralizing capacity were considered to contain equal amounts of virion antigen. Higher dilutions of materials from control cultures were required to get a similar reduction of neutralizing capacity; therefore, interferon inhibited the synthesis of serum-blocking antigens. The number of plaque-forming virions in the dilutions containing equivalent amounts of serum-blocking antigens was then calculated. The results of three experiments are tabulated in Table 4. In each case, the samples from the interferon-treated cultures containing the same amount of serum-blocking antigen as the samples from control

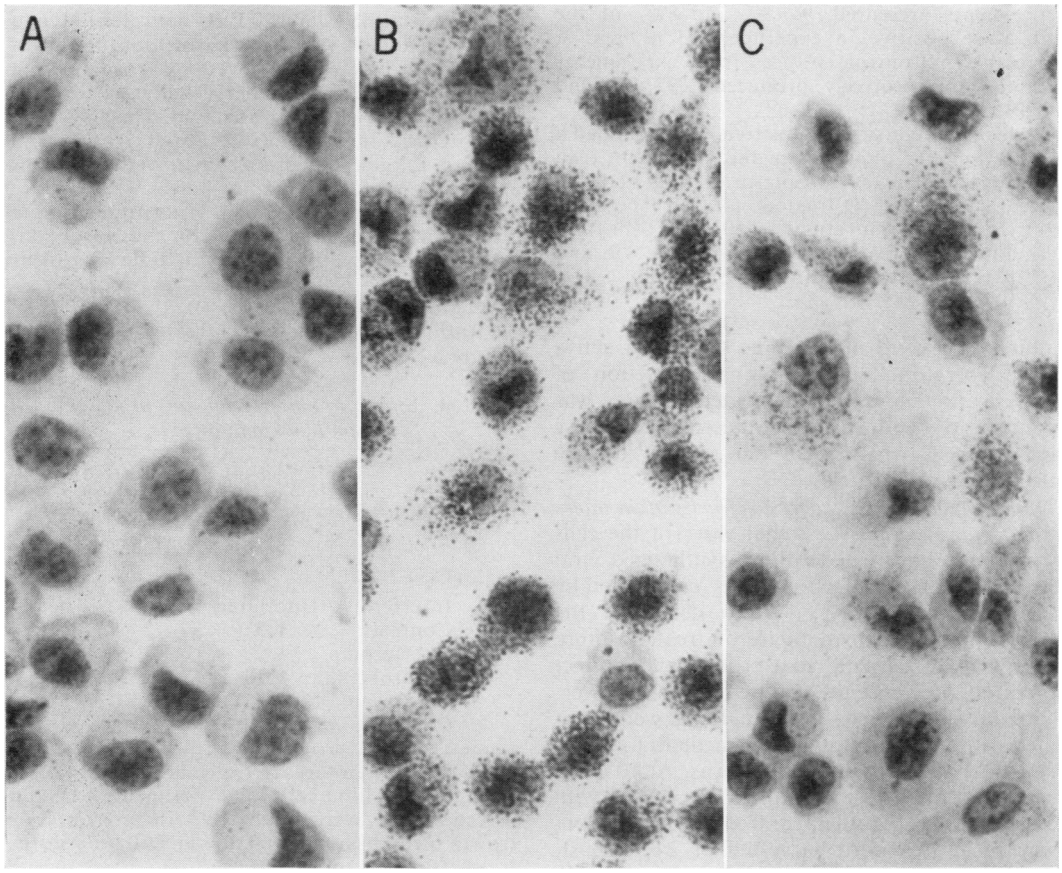

FIG. 2. *Photomicrographs of a radioautographic study on the effect of interferon on the incorporation of tritiated uridine into trichloroacetic acid-precipitable RNA. (A) Uninfected actinomycin D-treated (10 μg/ml) cell cultures. (B) Mengo virus-infected actinomycin D-treated cell cultures. (C) Interferon-treated Mengo virus-infected and actinomycin D-treated cell cultures.* × *557.*

TABLE 2. *Radioautographic analysis of synthesis of viral RNA in interferon-treated and control cells*[a]

Expt no.	Sample	No. of cells counted	No. of cells positive[b]	Cells positive (%)	Virus yield (%)
1	Interferon	622	214	36	11
	Control	621	593	100	100
2	Interferon	318	105	37	14
	Control	268	240	100	100
3	Interferon	340	84	28	11
	Control	320	286	100	100

[a] Monolayer cultures of 1.5, 1.2, and 1.6 million cells were incubated with interferon or growth medium for 16, 18, or 20 hr prior to infection with 20 PFU per cell of Mengo virus in experiments 1, 2, and 3, respectively. The cover-slip cultures were fixed at 7, 8, and 11.5 hr with methanol at 0 C in experiments 1, 2, and 3, respectively. Monolayer cultures from each experiment were harvested at 20 to 24 hr postinoculation for virus yields. In the experiments presented, 96, 90, and 89% of the cells were positive in experiments 1, 2, and 3, respectively. Control cultures from experiments 1, 2, and 3, respectively, produced 1,292, 362, and 543 PFU per cell.

[b] A cell was considered positive if it contained a greater number of grains in the cytoplasm than were observed in the cytoplasm of cells of uninfected actinomycin D-treated cultures. The average cytoplasmic grain counts of 50 to 100 cells from actinomycin D-treated cultures were 6.9, 4.2, and 13.7 in experiments 1, 2, and 3, respectively.

cultures contained about three times less active virus. Therefore, the additional reduction of neutralization in interferon-treated cultures was assumed to be due to the presence of excess viral antigens which were not part of mature infectious virus particles.

Immunofluorescence analysis of cells in an interferon-treated culture. Less than 40% of the cells in an interferon-treated culture synthesized viral RNA (Table 2) or infectious virus, as detected by infectious-center assays (9). To determine the proportion of cells in an interferon-treated culture which synthesized viral coat protein, the indirect immunofluorescence test was performed on cover-slip cultures of L cells. These cultures were incubated with interferon or growth medium for 16 hr prior to challenge with Mengo virus. At 10 or 11 hr postinoculation, the cultures were fixed with acetone at 0 C. Similar control and interferon-treated cultures were harvested at 24 hr postinoculation, and the virus yields were determined.

Included in the photomicrographs showing typical results (Fig. 3), an uninfected cell control (Fig. 3A) is shown. A low level of nonspecific cell fluorescence was observed in the cell control, especially in cells which had rounded up. The green, nonspecific fluorescence was discernible from the bright-yellow immunofluorescence. The infection of L cells with Mengo virus resulted in a majority of the cells exhibiting immunofluo-

TABLE 3. *Effect of interferon on production of Mengo virus hemagglutinins*[a]

Expt no.	Sample[b]	HAU[c]	PFU/ml $\times 10^7$	PFU/HAU $\times 10^6$	PFU/HAU[d]
1	Interferon	60	6.5	1.1	1.8
	Control	640	44.0	0.6	
2	Interferon	80	21.0	2.6	1.5
	Control	1,280	220.0	1.7	
3	Interferon	20	9.2	2.6	1.0
	Control	480	203.0	2.7	

[a] Monolayer cultures of Lts cells containing 1.8, 2.5, and 2.3 million cells in experiments 1, 2, and 3, respectively, were incubated with interferon or growth medium for 16 hr. Each culture was washed with PBSA, and 20 PFU per cell of Mengo virus was added. After the adsorption period of 1 hr, free virus was removed by washing with PBSA. All cultures were harvested at 16 to 18 hr by scraping the cells into the fluid. The cells were disrupted by three cycles of rapid freezing and thawing. Large cell debris was removed by centrifugation at 700 $\times$ *g* for 5 min.

[b] In experiments 1, 2, and 3, virus production in interferon-treated cells was 15, 10, and 5% of control values, respectively. Harvest time was 18 hr postinoculation.

[c] Hemagglutinin unit.

[d] Ratio between interferon-treated and control cultures.

TABLE 4. *Effect of interferon on production of serum-blocking antigens*[a]

Expt no.	Test sample[b]	Dilution of test sample	Reduction in neutralization by test samples (%)	No. of PFU in test sample $\times 10^7$
1	Interferon	Undiluted	70	4.6
	Control	1:5	72	16.2
2	Interferon	1:2	45	4.9
	Control	1:10	52	14.0
3	Interferon	1:4	41	3.5
	Control	1:10	43	11.0

[a] Monolayer cultures of Lts cells containing 1.8, 2.2, and 2.3 million cells for experiments 1, 2, and 3, respectively, were incubated with interferon or growth medium for 16 hr. Subsequent procedures are the same as in Table 2.

[b] In experiments 1, 2, and 3, respectively, virus production in the interferon-treated cultures was 6, 7, and 13% of control values. Control curves produced 935, 1,350, and 965 PFU per cell, respectively, in experiments 1, 2, and 3. Harvest time was 18 hr postinoculation.

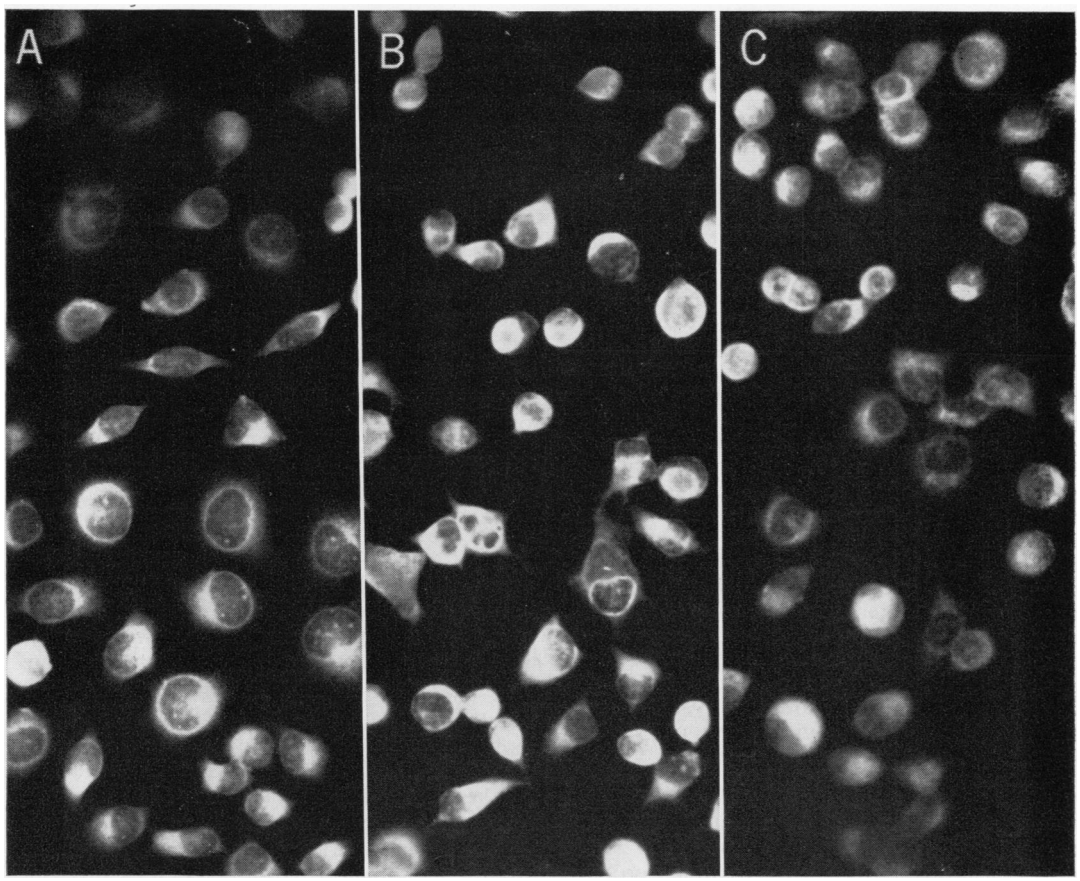

FIG. 3. *Photomicrographs of immunofluorescent studies. (A) Uninfected cell control. (B) Virus-infected cells. (C) Interferon-treated virus infected cells.* × *270.*

rescence (Fig. 3B). The incubation of cells with interferon prior to infection clearly reduced the number of cells showing immunofluorescence (Fig. 3C). The results are expressed quantitatively in Table 5. In the infected cells (control cultures), 81 to 86% of the cells were immunofluorescent-positive. If the control values are taken as 100%, interferon reduced the number of immunofluorescent-positive cells to 36 to 47% of control values.

DISCUSSION

All the cells in an interferon-treated culture of L cells are destroyed after infection with Mengo virus. Virus production is greatly reduced, and the proportion of cells able to act as infectious centers is greatly reduced (9). Therefore, the present study was guided by the following two questions. (i) Are detectable virion components produced in Mengo virus-infected cultures which have been previously incubated with interferon? (ii) Is there a correlation between production of a virion component and cell destruction?

Interferon, at concentrations which produced maximal reductions of Western equine encephalomyelitis virus yields (7), effected a similar level of reduction in yields of both mature virus and infectious viral RNA. Lockart and Sreevalsan (14) found a similar correlation for the replication of Western equine encephalomyelitis virus in interferon-treated chick embryo cells. Ho (10), however, found that interferon inhibited the synthesis of Eastern equine encephalomyelitis viral RNA much more than yields of infectious virus. Mecs et al. (16) reported that interferon reduced yields of Semliki Forest virus in chick embryo cells by 92 to 99%, but yields of infectious RNA by only 67 to 81%. In the present studies, since a low level of infectious RNA was synthesized by interferon-treated cultures, it was important to determine whether some or all cells in the culture were active in this synthesis.

The percentage of cells synthesizing viral RNA in interferon-treated and control cultures was determined by radioautographic techniques. Since the proportion of cells which synthesized

TABLE 5. *Immunofluorescence analysis of Mengo virus-infected, interferon-treated and control cells*[a]

Expt no.	Sample	No. of cells counted	No. of cells positive[b]	Positive cells (%)	Virus yield (%)
1	Interferon	505	152	36	20
	Control	511	424	100	100
2	Interferon	659	250	47	9
	Control	710	575	100	100
3	Interferon	380	137	42	17
	Control	406	350	100	100

[a] Monolayer cultures of 1.0, 1.2, and 1.1 million cells were infected with 20 PFU per cell of Mengo virus following incubation with interferon or growth medium for 20, 23, and 18 hr, respectively, in experiments 1, 2, and 3. The cover-slip cultures were fixed at 10 or 11 hr with acetone at 0 C, and the indirect immunofluorescence test was performed. In experiments 1, 2, and 3, respectively, 84, 81, and 86% of the cells were immunofluorescent-positive. Control cells produced 600, 1,640, and 373 PFU per cell in experiments 1, 2, and 3, respectively.

[b] Immunofluorescent-positive cells were characterized by bright yellow fluorescence. Negative cells exhibited a green fluorescence. All observations were performed at a magnification of 270 ×.

viral RNA (28 to 37%) in an interferon-treated culture was nearly the same as the proportion of cells which act as infectious centers (31 to 34%), these are probably the only cells which produce virus. Destruction therefore must occur in cells, even though they synthesize no viral RNA. Since new protein synthesis is required for cell destruction (2, 9), the input viral genome(s) must code for a protein(s) which leads to cell death. The input genomes, per se, are not responsible for cell death, since irradiated virus does not kill the cell (9).

The production of hemagglutinin in interferon-treated cultures was inhibited to the same extent as replication of infectious virus. This finding suggests that hemagglutinin merely reflected the number of virus particles produced. The synthesis of serum-blocking antigens in interferon-treated cells was reduced also, but there was consistently a threefold excess of these proteins above the level expected for the number of PFU produced. The inhibition of synthesis of viral proteins by interferon is probably a reflection of the reduced level of viral RNA molecules which serve as templates for the synthesis of viral proteins (22). Viral enzymes have been inhibited also. Miner et al. (17) demonstrated that interferon-treated L cells exhibited a reduced amount of Mengo viral RNA polymerase activity. They showed that the reduction of activity was not due to an inhibitor of the polymerase brought about by incubation with interferon. Similar results on the inhibition of synthesis of Semliki Forest virus RNA polymerase from interferon-treated chick embryo cells were reported (20).

The immunofluorescence studies showed that only 36 to 47% of the cells contained detectable amounts of coat proteins. This finding does not support the hypothesis that the threefold excess amount of protein (by the serum-blocking test) is distributed among the cells in interferon-treated cultures. However, the present data cannot unequivocally rule out the hypothesis for two reasons. (i) The level of coat proteins required for lethality may be below the level of detection of the immunofluorescence technique employed, and, therefore, low lethal levels of these proteins might be synthesized in the nonvirus-producing cells. (ii) Lethal levels of coat protein are produced which are not in the proper configuration to react with antibody against mature virus.

Amako and Dales (2) and Gauntt and Lockart (9) have shown, in the Mengo virus–L cell system, that protein synthesis must occur near the end of the latent period for cell destruction to ensue. Bablanian et al. (3) reported similar findings for the poliovirus-human embryonic lung (diploid) system. Amako and Dales (2) and Bablanian et al. (3) interpreted this protein synthesis to mean synthesis of capsid proteins, since this is the time that capsid protein can first be detected. However, the additional data from the immunofluorescence studies lend support to the hypothesis that cell destruction is due to a protein which is not part of the mature virus. The hypothesis would therefore propose that the input Mengo genomes code for a protein(s) while under the influence of the action of interferon which is responsible for cytotoxicity. The hypothesis does not seem to be in direct conflict with present hypotheses on the mode of action of interferon. By using a virus which is highly susceptible to the action of interferon, Marcus and Salb (15) showed that, in a cell-free system, Sindbis virus RNA attaches to ribosomes from interferon-treated chick embryo cells with two-thirds the normal efficiency, and apparently the viral RNA is not translated. Other studies with viruses less sensitive to interferon have suggested that interferon acts by preventing polyribosome formation (4, 12). However, in both of these studies, a small but detectable level of polyribosome formation occurred, and this level might be sufficient for synthesis of cytotoxic protein(s).

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LITERATURE CITED

1. Adams, M. H. 1959. Bacteriophages. Interscience Publishers, Inc., New York.
2. Amako, K., and S. Dales. 1967. Cytopathology of Mengo virus infection. 1. Relationship between cellular disintegration and virulence. Virology **32**:184–200.
3. Bablanian, R., H. J. Eggers, and I. Tamm. 1965. Studies on the mechanism of poliovirus-induced cell damage. II. The relationship between poliovirus growth and virus-induced morphological changes in cells. Virology **26**:144–121.
4. Carter, W. A., and H. B. Levy. 1967. Ribosomes: effect of interferon on their interaction with rapidly labeled cellular and viral RNA's. Science **155**:1254–1258.
5. De Somer, P., A. Prinzie, P. Denys, and E. Schonne. 1962. Mechanism of action of interferon. I. Relationship with viral ribonucleic acid. Virology **16**:63–69.
6. Dulbecco, R., and M. Vogt. 1954. One-step growth curve of Western equine encephalomyelitis virus on chicken embryo cells grown *in vitro* and analysis of virus yields from single cells. J. Exptl. Med. **99**:183–199.
7. Franklin, R. M. 1962. A cytochemical description of the multiplication of Mengo virus in L-929 cells. J. Cell Biol. **12**:1–15.
8. Franklin, R. M., and D. Baltimore. 1962. Patterns of macromolecular synthesis in normal and virus-infected mammalian cells. Cold Spring Harbor Symp. Quant. Biol. **27**:175–198.
9. Gauntt, C. J., and R. Z. Lockart, Jr. 1966. Inhibition of Mengo virus by interferon. J. Bacteriol. **91**:176–182.
10. Ho, M. 1963. Effect of an interferon on synthesis of viral ribonucleic acid and plaque formation. Proc. Soc. Exptl. Biol. Med. **112**:511–515.
11. Homma, M., and A. F. Graham. 1963. Synthesis of RNA in L cells infected with Mengo virus. J. Cellular Comp. Physiol. **62**:179–192.
12. Joklik, W. K., and T. C. Merigan. 1966. Concerning the mechanism of action of interferon. Proc. Natl. Acad. Sci. U.S. **56**:558–565.
13. Lockart, R. Z., Jr. 1964. The necessity for cellular RNA and protein synthesis for viral inhibition resulting from interferon. Biochem. Biophys. Res. Commun. **15**:513–518.
14. Lockart, R. Z., Jr., and T. Sreevalsan. 1963. The effect of interferon on the synthesis of viral nucleic acid, p. 447–461. *In* Virus, nucleic acids and cancer. The Williams & Wilkins Co., Baltimore.
15. Marcus, P. I., and J. M. Salb. 1966. Molecular basis of interferon action: inhibition of viral RNA translation. Virology **30**:502–516.
16. Mecs, E., J. A. Sonnabend, and E. M. Martin. 1967. The effect of interferon on the synthesis of RNA in chick cells infected with Semliki Forest virus. J. Gen. Virol. **1**:25–40.
17. Miner, N., W. J. Ray, Jr., and E. H. Simon. 1966. Effect of interferon on the production and action of viral RNA polymerase. Biochem. Biophys. Res. Commun. **24**:264–268.
18. Reich, E., R. M. Franklin, A. J. Shatkin, and E. L. Tatum. 1961. Effect of actinomycin D on cellular nucleic acid synthesis and virus production. Science **134**:556–557.
19. Scherrer, K., and J. E. Darnell. 1962. Sedimentation characteristics of rapidly labeled RNA from Hela cells. Biochem. Biophys. Res. Commun. **7**:486–490.
20. Sonnabend, J. A., E. M. Martin, and E. Mecs. 1967. The effect of interferon on the synthesis and activity of an RNA polymerase isolated from chick cells infected with Semliki Forest virus. J. Gen. Virol. **1**:41–48.
21. Taylor, J. 1964. Inhibition of interferon action by actinomycin. Biochem. Biophys. Res. Commun. **14**:447–451.
22. Tobey, R. A. 1964. Mengo virus replication. II. Isolation of polyribosomes containing the infecting viral genome. Virology **23**:23–29.
23. Tobey, R. A., and E. W. Campbell. 1965. Mengo virus replication. III. Virus reproduction in Chinese hamster ovary cells. Virology **27**:11–16.