Variation of Interferon Production During the Cell Cycle

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The capacity of cells to produce interferon has been found to depend on the phase in the cell cycle at which virus infection took place. Monolayer cultures of L cells were synchronized by the double thymidine-block method. Such synchronously growing cultures were used to study the ability of cells to produce interferon when they were infected with ultraviolet-inactivated Newcastle disease virus (UV-NDV) at different phases of the cell cycle. In all instances, interferon was detected early and reached a maximum at about 16 hr after infection. However, the levels of interferon found in medium of cultures infected at early post-deoxyribonucleic acid (DNA) synthetic (G2) and to some extent at late G2 phases of the cell cycle were comparatively lower than those found in cultures infected at the early DNA synthetic (S) phase. There appeared also in these infected growing cultures a transient period when interferon production was apparently delayed. This period corresponded interestingly with the time of mitotic burst. Infection of thymidine- or 1-β-D-arabinofuranosylcytosine-inhibited cultures with UV-NDV also led to similar interferon response as that observed in growing cultures infected at early S. However, no transient delay of interferon production was demonstrated in these cultures.

There is evidence that the general state of control of cellular synthetic processes at a particular time may affect interferon synthesis. There is marked difference in the ability of leukocytes to respond to induction of interferon depending on whether they were drawn from normal or leukemic individuals (6, 9). There is also a difference in the magnitude of the response to induction depending on the type of leukemia being suffered by the leukocyte donor (10). Friedman (4) has shown an inverse relationship between overall protein synthesis and interferon synthesis. Some conditions of growth which favored a higher than normal rate of synthesis of cell protein resulted in low interferon yields. In other reports (15, 16) in which other specialized proteins were studied, it was found that conditions that favored proliferation adversely affected synthesis of these proteins. A major difficulty in gathering unequivocal information on interferon induction exists because cell cultures used in studies have been growing asynchronously and various proportions of the cell population are in different control situations as befits their physiological state at different phases of the life cycle.

Reported here are the results of experiments in which synchronized cultures of mammalian cells were induced to produce interferon at various phases of the cell cycle and after induction the rate of interferon synthesis was measured over a period of time.

MATERIALS AND METHODS

Cell cultures. Primary chick embryo fibroblast (CEF) monolayers were prepared from 10-day-old embryonated eggs. Cells were grown in medium containing Gey's balanced salt solution with 0.0025 M tris(hydroxymethyl)aminomethane, 0.25% lactalbumin hydrolysate, and 0.1% proteose peptone supplemented with 5% inactivated calf serum as described by Gifford et al. (5). Mouse L cells (strain L-929) were grown in Eagle's minimal essential medium supplemented with 10% inactivated fetal calf serum (FCS). A clonal isolate of this cell line was prepared and included in the experiments. When grown in 60-mm petri dishes, L-cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO2 in air.

Viruses. Newcastle disease virus (NDV), California strain, was purchased from the American Type Culture Collection. The virus, grown in the allantois of 10-day-old fertile eggs, was harvested 72 hr after infection and was assayed by plaque formation on 2-day-old monolayer cultures of CEF. For interferon induction, ultraviolet (UV)-inactivated NDV (UV-NDV) was used. UV irradiation was performed by
exposing a 3-ml sample of virus fluid [initial titer of log$_2$ 9 plaque-forming units (PFU)/ml] in an open 60-mm petri dish at a distance of 25 cm from a General Electric 15-w germicidal lamp for 150 sec. During the period of irradiation, the virus fluid was placed on a shaker and agitated continuously. Absence of infectivity of the UV-NDV preparation was ensured by inoculating undiluted and suitably diluted samples into chick embryos and CEF cultures.

Vesicular stomatitis virus (VSV) used in interferon assay was propagated and assayed by the plaque technique in L-cell monolayers.

**Virus assay.** Two-day-old confluent CEF or L-cell monolayers grown in 60-mm petri dishes were used for virus plaque assay. The virus inoculum of 0.2 ml per dish was allowed to adsorb for 1 hr at 37°C, after which 5 ml of overlay medium was added. The overlay medium consisted of the respective basic medium for each cell type plus 2% FCS and 0.6% agarose. At 2 days postinoculation, each culture received 3 ml of the same overlay plus 0.003% neutral red. Plaques were counted after an additional 2 to 3 hr of incubation at room temperature in the dark.

**Cell synchronization.** Monolayer cultures of L cells grown in 60-mm petri dishes or on cover slips placed in Leighton tubes were synchronized by double treatment with excess thymidine (1, 11). In brief, the growth medium of 1- to 2-day-old cultures containing incomplete monolayers was replaced with the same medium containing excess thymidine (0.625 mg/ml). After 18 hr, the cultures were washed four times in the normal medium and incubated again at 37°C for 20 hr. This treatment with excess thymidine was then repeated, and after 18 hr the cultures were again washed and refed with growth medium. All petri dish cultures were incubated at 37°C in an environment of controlled humidity and CO$_2$ pressure. Replacement of medium and washing procedures were performed as fast as possible in a 37°C warm room.

After the removal of the second thymidine block, the degree of synchrony of the cells was determined by measuring the incorporation of a labeled deoxyribonucleic acid (DNA) precursor and also by the assessment of mitotic indexes at different time intervals.

**Radioactivity determination.** Two types of labeling experiments were employed throughout the course of study. In the first, the cells on cover slips were exposed to $^3$H-thymidine (1 $\mu$g/ml; 1.9 c/mmole, Schwartz BioResearch) for 30 min, after which the cells were washed in chilled phosphate-buffered saline (PBS) and fixed in an acetic acid-alcohol (1:3) mixture. To remove unincorporated $^3$H-thymidine, the cover slips were treated with 2% perchloric acid at 4°C for 40 min (11). Labeling of cells was detected by means of dipping autoradiography by using Ilford K2 emulsion. The exposure time was 5 days. Giemsa or hematoxylin and eosin stain was employed after autoradiography. These preparations were also used for the determination of mitotic indexes; only cells in metaphase were counted. For each time interval, at least 1,000 cells were examined.

In the second method, petri dish-cell cultures were similarly pulse-labeled with thymidine-2-$^3$H (TdR-$^3$H; 0.05 $\mu$C/ml; 53.6 mc/mmole, Schwartz Bio Research), after which the cells were washed with PBS and removed from the dish by 1-min treatment with 0.25% trypsin. The cell suspension was divided into three portions for total cell count in a hemocytometer, determination of mitotic indexes, and measurement of TdR-2-$^3$H uptake, respectively. The latter was performed by determining the incorporation of TdR-2-$^3$H into trichloroacetic acid-precipitable material by using the liquid scintillation counting technique as described (8). Total cell count and the estimate of mitotic indexes were performed by using the conventional citric acid-violet and Giemsa stains, respectively.

**Interferon production.** At various times after the cells were released from the thymidine block, they were induced to produce interferon. UV-NDV was used as an inducer at a multiplicity of 20 to 30 PFU. The killed-virus inoculum was prepared in growth medium and was adsorbed to cells for 1 hr at 37°C. The inoculum containing the unadsorbed virus was then removed, and the cultures were washed gently twice and placed in fresh growth medium at 37°C. At different hours after induction, two cultures were removed from the incubator and the culture fluid was pooled for interferon assay. The cells remaining in the petri dishes were trypsinized and the resulting suspension was used for total cell count.

**Interferon assay.** Prior to assay all interferon preparations were acidified to pH 2 with 1 N HCl for 24 hr at 4°C, neutralized with 1 N NaOH, and then subjected to ultracentrifugation at 100,000 $\times$ g for 3 hr in an International ultracentrifuge (model B60). Interferon assays were carried out on 2-day-old confluent monolayer cultures of L cells grown in 60-mm petri dishes. Each culture received 3 ml of the test fluid in various dilutions by using duplicate cultures per dilution. Control cultures were similarly set up with medium alone. After 16 hr of incubation, all cultures were challenged with approximately 100 PFU per culture of VSV in 0.2-ml amounts by the virus assay technique described above. Interferon titer was expressed as the reciprocal dilution at which virus plaque counts were reduced by 50% of that in control cultures (PDD$_{50}$; 13). As a common laboratory practice, a batch of L-cell interferon was prepared and stored at 4°C for not more than 3 months. It was included as a standard reference interferon in each series of assay to be carried out. The per cent standard deviation from six replicate determinations of the same preparation was ± 18.

**RESULTS**

In preliminary experiments, monolayer cultures of uncloned L cells were synchronized by double-block treatment with 0.625 mg of thymidine per ml. The degree of synchrony was determined by the relative proportion of cells showing $^3$H-thymidine labeling or in mitosis. The main characteristics of the data after thymidine removal were a period of increase in per cent labeled cells from 0 to 3 hr, a period of decline from 3 to 7 hr, a
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Fig. 1. Synchronization of cloned L cells by the double thymidine-block method. The degree of synchrony was determined by the uptake of thymidine-2-\(^{14}\)C into acid-precipitable cell constituents and by metaphase counts at various intervals after release from the second thymidine block.

A period of mitotic activity between 7 and 10 hr, and a period of leveling off in per cent labeled cells occurring around the 7th hr. These periods, as similarly illustrated in Fig. 1, were not absolutely delineated. Nevertheless, they would seem to correspond, respectively, to the phases of DNA synthesis (S), post-DNA synthesis (G2), mitosis (M), and postmitosis (G1) of the cell cycle.

In the same series of experiments, portions of the cultures taken immediately and at 3 hr after the second thymidine removal were induced to produce interferon by infection with UV-NDV. These intervals corresponded, respectively, to the early S and early G2 phases of the cell cycle. Three, 6, 9, and 15 hr after induction, interferon production was determined. The results from a series of three experiments similarly demonstrated that cells induced during early S consistently produced yields of interferon in the order of two- to threefold higher than that induced during early G2.

Stimulated by the above observation, two identical experiments were performed in an attempt to follow interferon production in synchronized cultures of cloned L cells at relatively closer intervals than before. Comparable results were noted in these experiments.

The results presented in Fig. 1 represent a typical experiment carried out as a control for the interferon study to be presented in Fig. 2. As has been noted earlier, after treatment with excess thymidine, a population of cloned L cells traversed a series of phases of activity in the cell cycle as judged both by the pattern of TdR-2-\(^{14}\)C incorporation into DNA and by the time and magnitude of the mitotic burst.

The comparative rate of interferon production stimulated at various phases of the cell cycle is presented in Fig. 2. Included in the figure also are the cell count data obtained by averaging the total cell number among the control and interferon cultures at different time intervals after thymidine removal and interferon induction. Thus, a comparison of cell number and interferon yield can be made. As can be seen, regardless of the time of induction, interferon was detected early and reached a maximum at the time preceding the next S phase (see also Fig. 1). There appeared, however, a transient period approximately 7 to 11 hr after the removal of the thymidine block when interferon production was apparently delayed. The data in Fig. 1 revealed that this period was occupied by cells traversing the late S, M, and early G1 phases of the cell cycle.

Fig. 2. Comparative rates of interferon production in synchronously growing cultures of cloned L cells. Cultures were infected with UV-NDV at 0 hr (○), 3 hr (□), and 5 hr (△) after thymidine release. Each point of the cell count data (△) represents the mean value from at least four cultures.
Figure 2 also shows that the yields of interferon in cultures induced during the early G2 were consistently lower than that induced in early S as described before. On the other hand, when cells apparently in the late G2 were induced to produce interferon, they produced yields which started relatively low, as did those induced in early G2, but subsequently ended up making comparable levels per culture as those induced in early S.

On two separate occasions, similar attempts were made to study interferon production in cells prevented from entering into or prevented from completing the S phase. This was accomplished either by maintaining the second thymidine block during virus adsorption and after infection or by exposing the cells to 15 μg of 1-β-D-arabinofuransylcytosine per ml (8) 2 hr prior to and immediately after the removal of the thymidine block and also during virus infection. In both instances, the culture fluids to be assayed for interferon were dialyzed against three changes of Eagle’s MEM at 4°C over a period of 72 hr to remove both chemicals. It is found that the time course of interferon production in these cultures was similar to that described in Fig. 2 for stimulation during early S. Maximum yields of interferon were reached at about the 16th hr after stimulation, and no apparent delay was noted over the period studied. Cell count determinations performed in these and in control cultures showed no significant increase in cell number per culture throughout the course of experiment.

DISCUSSION

The present studies have been made possible by the double thymidine-block technique for securing populations of uncloned and cloned L cells in highly synchronized condition. Utilizing such a method, the possible effects of the phase of the growth cycle on interferon production stimulated by UV-NDV was investigated. For convenience, the time intervals chosen for stimulation represented early S, late S-early G2, and late G2 phases of the cell cycle. Preliminary results indicated the following.

(i) The levels of interferon detected in the medium of synchronously growing cultures infected during early G2 and, to some extent, during late G2 phases of the cell cycle were lower than those found in the medium of growing cultures infected during early S phase. The reason for this variation is not clear. Of interest is the finding (2) that the rate of ribonucleic acid (RNA) synthesis is much higher during G2, which includes late interphase after DNA synthesis and prophase, than during early and mid-interphase (G1 and early S). Enger, Tobey, and Saponara (3) also reported a much higher rate of ribosomal RNA methylation in G2 than in G1. It is thus probable that an efficient expression of the transcriptional and translational events of interferon synthesis may depend on the overall cell cycle pattern of RNA synthesis.

(ii) A surprising finding was that, although cells infected in early and late G2 phases both yielded low levels of interferon in early hours after infection, yet those late in G2 produced substantially higher yields than those cultures infected in early G2. Indeed, the yields were as high as those infected in early S. A possible explanation would be that more cells might become available for the initial induction process of interferon synthesis as the infected late G2 cells completed division. This would be compatible with the cell count and mitosis data presented in Fig. 1 and 2.

(iii) We do not know precisely what phase of the cell cycle may be responsible for the transient delay of interferon production noted approximately 7 to 11 hr after the release of the second thymidine block (Fig. 2). The data in Fig. 1 revealed that this period was occupied by cells in late G2, M, and early G1 phases. The occurrence of mitotic burst during this period suggests the possibility that cellular mitotic activity may play a role in limiting the production of interferon. Very recently, a decrease in protein synthesis significantly associated with the disaggregation of polyribosomes has been demonstrated in HeLa cells during cell division by Hodge, Robbins, and Scharff (7). Their findings further indicated that some of the RNA present in late G2 persisted through mitosis and continued to function as the cells reentered interphase. Moreover, colchicine, a potent substance of mitotic arrest, has been shown (14) to lower the activity of viral interference in mice and chick embryo cell cultures and also to decrease the synthesis of interferon in chick embryos.

The results reported thus far are largely preliminary. Whether the sequential transcriptional and translational events of interferon production during the cell cycle are controlled by the homeostatic mechanism of the host remains to be elucidated. Further study along this line with a synchronous system with a high degree of mitotic synchrony, such as that by the application of nitrous oxide under pressure (12), may be of value. In addition, a knowledge of the relative adsorption of virus to cells in various phases of the cell cycle would be valuable in the interpretation of the findings.

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