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The Effect of Cell Density on the Production of Interferon[1] (37825)

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(Introduced by D. A. Levy)

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Certain similarities between interferon formation and induced enzyme synthesis (1–3) have evoked the hypothesis that virus-induced interferon synthesis involves the derepression of an interferon gene (3, 4). Evidence for this consists of the inhibition of interferon production by actinomycin D (1, 5) and the lack of spontaneous synthesis of interferon in most cell systems that have been studied with a few exceptions (6–8). Initiation by viruses or other inducers is usually needed for interferon production.

The capacity of cells to synthesize interferon is related to their metabolic state (9). Increasing the rate of protein synthesis by various methods results in a decrease in the production of interferon (10). This has been explained by postulating that an increased amount of repressor is produced under those conditions, rendering the cells more refractory to induction (10).

The present report compares the production of interferon in L-929 cells growing under different culture conditions, thereby providing further evidence that the density of cells and their metabolic state influence their production of interferon.

Materials and Methods. L-929 (L) cells, a line of mouse fibroblasts (Flow Lab.), were grown in monolayer in 75-cm^2 Falcon plastic flasks using Eagle's minimum essential medium (MEM) (Baltimore Biological Labs) supplemented with 15% fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 U/ml). When the monolayers were confluent, they were infected at a multiplicity of 5 plaque-forming units (PFU) per cell with Newcastle Disease Virus (NDV) (California strain) which was prepared in chick allantoic fluid. The excess virus was aspirated after agitation for 1 hr at 37°, and the monolayers were washed once with MEM. The flasks were then divided into two groups of equal numbers, one group for monolayer and the other for spinner culture. The spinner and monolayer cultures were then compared according to cell counts, NDV replication, interferon, and incorporation of radioactive precursors.

The monolayers for spinner were dispersed with a 0.05 *M* solution of ethylenediamine tetraacetic acid (EDTA) (Grand Island Biological Co.) in order to avoid the proteolytic effect of trypsin. The cells were pooled with a final concentration of 5×10^5 cells/ml in MEM spinner balanced salt solution (Baltimore Biological Labs) supplement with 10% fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 U/ml) and placed in a 500-ml single sidearm flask which contained a nonheating magnetic stirrer (Bellco Biological Glassware, Vinelans, NJ). The flasks set aside for monolayer culture received the same medium (including EDTA, which was added to the medium) as the spinner culture. The total volume of medium in both groups was equal. The total number of cells in both groups were also the same, with

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each group numbering 10^8 cells.

The plastic flasks containing the monolayer of cells and the spinner flask containing the cell suspension were then placed in a stainless steel desiccator (Boekel Company, Philadelphia, PA) and incubated at 37° under 5% CO_2 humidified air. The desiccator was placed on a tempco-stir plate (Thermolyne Corporation, Dubuque, IA) and the current set so that the magnetic stirrer in the spinner flask rotated approximately 200 rpm. Cell counts were done from the spinner flask at 24-hr intervals, and aliquots were taken from spinner and monolayer flasks at the beginning and end of the incubation period for NDV titers which were assayed on chick embryo cell monolayers.

At the end of 48 hr, the media from the plastic flasks and spinner flasks were processed for interferon as previously described (9). In several experiments, media were also removed from the flasks at interval periods from 2 to 48 hr to establish the kinetics of interferon production under these conditions. The assay for interferon consisted of a plaque reduction technique in L cells employing vesicular stomatitis virus (VSV) as the challenge virus. The interferon titer was calculated as the greatest dilution which produced a 50% reduction in the number of plaques (PR_{50}).

At intervals during the time interferon production was being assessed, the incorporation of radioactive precursors was compared in cells from monolayer and spinner cultures using methods previously described (11). For the monolayer culture, one flask containing 1×10^7 cells, and separate from those used for the interferon production, was incubated for 15 min at 37° with 5 ml of ^{3}H-methylthymidine (500 μCi/ml, 18.35 Ci/mmole; New England Nuclear Corp. and 20 ml of ^{14}C-leucine (100 μCi/ml, 327 Ci/mmole; New England Nuclear Corp.). Following the incubation, the medium was decanted from the flask and replaced with cold phosphate-buffered saline (PBS) containing 1 ml each of 1 m*M* thymidine and 1 m*M* leucine. The monolayer was kept at 4° for 5 min, washed 3 times with PBS, the cells made into suspension with a solution of 0.05% trypsin–10% EDTA (Difco Labs), and finally collected by centrifugation at 10,000 rpm for 5 min.

For the spinner culture, an aliquot containing the same amount of cells as the monolayer flask was withdrawn and incubated with the same amounts of radioactive precursors as the monolayer. The cells were washed and pelleted in a similar manner as the monolayer.

After the cells from both monolayer and spinner cultures were pelleted, they were dissolved in 0.25 ml of formic acid and placed at 70° for 5 min. Radioactivity was determined by double-channel counting in a Nuclear-Chicago Unilux scintillation spectrophotometer with appropriate standards.

Results. The data in Table I clearly demonstrates that the culture conditions of cell growth influence the production of interferon. In the six separate experiments listed, the monolayer culture produced from 4- to 32-fold more interferon than that from the respective spinner culture. The viral inhibitor produced had features consistent with interferon being: stable at pH2, nondialyzable, species specific, trypsin sensitive, and ineffective in directly inactivating the virus. The differences observed were not due to simple mechanical trauma to the cells in spinner culture, since

TABLE I. Comparison of Interferon Production in Spinner and Monolayer Cultures.

Exp. No.	Growth conditions	PR_{50}[a] titers of interferon
1	Spinner	1:8
	Monolayer	1:256
2	Spinner	1:64
	Monolayer	1:256
3	Spinner	1:16
	Monolayer	1:256
4	Spinner	<1:4
	Monolayer	1:64
5	Spinner	<1:4
	Monolayer	1:128
6	Spinner	1:32
	Monolayer	1:512

[a] The greatest dilution of interferon which produced a 50% reduction in the number of plaques.

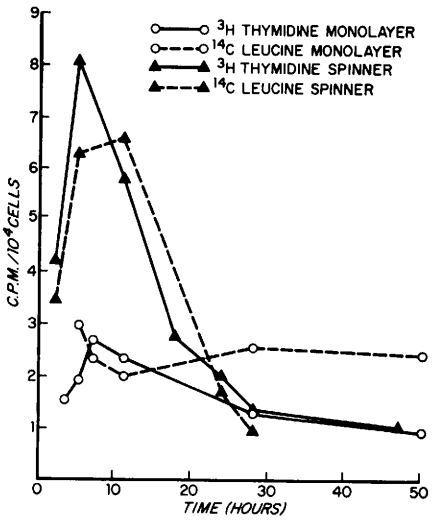

FIG. 1. Comparison of the incorporation of ^{3}H-thymidine and ^{14}C-leucine in uninfected monolayer and spinner cell cultures. Representative experiment.

it was established with noninfected L cells that they begin to show a burst of metabolic activity (Fig. 1) almost immediately after being placed in suspension, peaking at 6 hr and lasting for 18 hr. Over this period of time, the cell count increased 2–3-fold, with their viability demonstrated by the absence of staining with 0.5% trypan blue. These results of limited growth of L cells in suspension culture under conditions where the medium is not changed are similar to those previously reported (12).

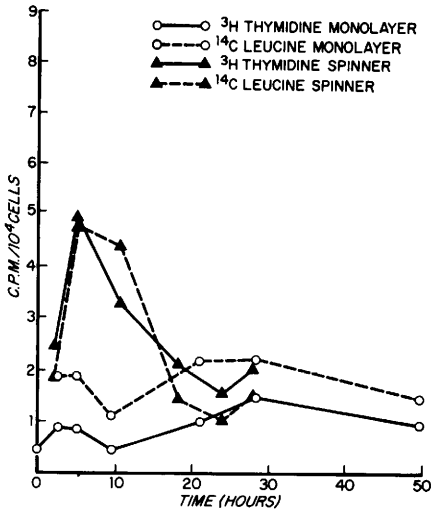

FIG. 2. Comparison of the incorporation of ^{3}H-thymidine and ^{14}C-leucine in infected monolayer and spinner cell cultures. Representative experiment.

In the infected spinner culture, the parameters that ruled out the possibility of mechanical damage were the cell counts remaining unchanged and that even though the amount of interferon produced differed from that of monolayer conditions, the titers (8×10^{3} PFU/ml) of inducing virus (NDV) produced by the monolayer and spinner cultures were similar. The infected spinner culture (Fig. 2) also demonstrated a burst of metabolic activity but to a lesser extent than the uninfected culture. This showed that the NDV infection only partially effected the metabolic activity of the cells, which may reflect the limited extent to which NDV replicates in L cells (13).

In addition to interferon production, spinner and monolayer cultures were different regarding their metabolic activity as measured by incorporation of ^{3}H-thymidine and ^{14}C-leucine. Figures 1 and 2 show that DNA and protein synthesis were increased in the spinner cultures for both cells infected with NDV and uninfected cells. In contrast, the monolayer cultures demonstrated minimal activity, as would be expected in cells that are arrested by contact inhibition (11, 14).

Discussion. The data presented demonstrates that under culture conditions where cell densities are different, there is a difference in interferon production and metabolic activity. An hypothesis that may explain the effect that cell density may have on the production of interferon involves derepression of an interferon gene in virus-induced interferon synthesis (3, 4). Recent evidence that cycloheximide, actinomycin D, and puromycin increased the interferon response to either uv-irradiated NDV or polyinosinic and polycytidylic acids in rabbit kidney cell cultures (depending on the time the drugs were added) suggests a mechanism whereby a control protein normally checks interferon production (15–17).

As cells in monolayer become confluent,

biochemical events accompany the development of contact inhibition which results in an overall decrease in macromolecular synthesis (11, 14). Growth conditions for spinner cultures preclude contact inhibition, and, hence, the spinner and monolayer cultures have different metabolic states, as was demonstrated. The data shows that the ability of cells to be induced to produce interferon appeared to be inversely related to their metabolic activity with the monolayer culture possessing a larger capacity for induction than that of the spinner culture. Support for a causal relation between the inverse correlation of these activities is suggested by the temporal relation of interferon production in these systems to the levels of metabolic activity. In both spinner and monolayer cultures, interferon production begins at 5 hr and peaks at 24 hr. The greatest differences in macromolecular synthesis between the spinner and monolayer cultures occur before and during early interferon production. These observations are similar to those reported previously (10) and are of further interest since cell density has been demonstrated to influence the action of interferon in chick embryo cells (18).

Summary. L-929 cells growing under different conditions of spinner and monolayer cultures were compared according to cell counts, NDV replication, interferon production, and incorporation of radioactive precursors. With equal numbers of cells and NDV replication, the monolayer culture produced 4–32-fold more interferon than that from the respective spinner culture. The spinner and monolayer cultures were also different regarding their metabolic activity as measured by the incorporation of ^{3}H-thymidine and ^{14}C-leucine. DNA and protein synthesis were increased in the spinner culture in contrast to the minimal activity of the monolayer culture. The data shows that the monolayer culture possessed a larger capacity for the induction of interferon than that of the spinner culture, which provides further evidence that the density of cells and their metabolic state influence their production of interferon.

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