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Influence of the Rate of Cell Growth and Cell Density on Interferon Action in Chick Embryo Cells[1]

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Chick embryo cells became more sensitive to the action of interferon the longer they remained in culture. This phenomenon was found even before confluency had been reached. The relative insensitivity of newly seeded cells was not due to a loss of receptors. Cells synthesizing deoxyribonucleic acid (DNA) at a high rate were less sensitive to interferon action than cells synthesizing DNA at a low rate, but the inhibition of DNA synthesis had no effect on interferon action. An increase in the number of cells used for seeding resulted in an earlier appearance of increased sensitivity to interferon action. These results are discussed in relation to the induction process in animal cells.

It has previously been reported that the capacities to produce and to respond to the action of interferon are influenced by the age of cells in culture. Cantell and Paucker (2) found that 1- to 3-day-old cultures of a HeLa cell line produce less interferon and are less sensitive to its action than are 6- to 10-day-old cultures. In chick embryo cell cultures, 6- to 8-day-old cells produce 5 to 18 times more interferon per cell than 1- to 2-day-old cells (8). Similar findings were reported by Carver and Marcus (3), who compared young and aged confluent monolayers of chick embryo cells. Recently, Lockart (13) showed that the rate at which the antiviral effect of interferon is conferred is faster in aged chick embryo cells.

Interferon seems to act indirectly by causing the synthesis of new ribonucleic acid (RNA) and protein (6, 21). Presumably, the new protein(s) is responsible for the antiviral effect, perhaps by attaching to ribosomes rendering them incapable of forming a functional polysome with viral RNA (12, 14). The production of interferon in response to a virus also requires new RNA synthesis (7). Thus, both the production and the action of interferon show a resemblance to enzyme induction. Since both processes are less efficient in young cells, it is possible that they are two examples of a more general phenomenon; any inducible process may be hampered by cell growth.

We have attempted to define the factors which are responsible for the increase in the sensitivity of chick embryo cells to interferon action with aging. We were particularly interested in ascertaining whether cell growth or cell density is involved in this phenomenon.

MATERIALS AND METHODS

Cell cultures. The methods, media, and solutions used in the preparation and cultivation of primary chick embryo cells have been described (23). Plastic flasks (250 ml; Falcon Plastic) were seeded with 20×10^6 cells in 25 ml of medium. Secondary cultures of chick embryo cells were obtained by trypsinization of 2- to 3-day old flasks of primary cultures, unless otherwise stated. They were usually seeded at 2.5×10^6 cells per petri dish (60 mm; Falcon Plastic). They reached a maximum density of approximately 6×10^6 cells per petri dish.

Viruses. The origin and method of plaque assay of vesicular stomatitis virus (VSV) were the same as indicated previously (23). Chikungunya virus was obtained from J. Casals, Yale University, New Haven, Conn. Stock 10% suspensions were prepared from brains of suckling Swiss Webster mice inoculated intracerebrally. Chikungunya virus was titrated by intracerebral inoculation of suckling mice. All virus stocks were kept frozen at −70 C.

Interferon. Interferon was produced in chick embryo cell cultures grown in flasks. Five- to 7-day-old cultures were inoculated with Chikungunya virus at a multiplicity of about 0.5 LD_{50} per cell. After inoculation, cells were maintained at 36 C in Eagle minimal essential medium (MEM) without serum. Tissue culture fluids were collected 24 hr after inocula-

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tion and were stored at 4 C until used. Infectious virus was inactivated by heating the interferon at 60 C for 1 hr. The interferon was titrated by the plaque inhibition method (24).

Virus yield assay. After removal of the fluids from the monolayer, VSV was added at a multiplicity of at least 3 plaque-forming units (PFU)/cell in 0.5 ml of MEM. The cultures were incubated for 1 hr and then were washed four times with Earle balanced salt solution (Grand Island Biological Co., Grand Island, N.Y.). Then 4 ml of MEM containing 2% gamma globulin-free calf serum was added, and the cultures were incubated at 36 C. Duplicate samples of the fluids were taken at 9 to 16 hr and stored at −25 C until titrated.

Deoxyribonucleic acid (DNA) synthesis. DNA synthesis was determined by the cover slip method (1). Glass petri dishes (60 mm) containing 3 cover slips (9 by 22 mm; Bellco Glass, Inc., Vineland, N.J.) were seeded with secondary chick embryo cells. DNA synthesis was measured by a 1-hr pulse with ^{3}H-thymidine (Nuclear-Chicago Corp., Des Plaines, Ill., 5,000 mc/mM), adding 0.2 ml of a 20 μc/ml solution to 2 ml of medium. At the end of the pulse, the medium was removed and cultures were flooded with ice-cold acetic acid-ethyl alcohol (1:3). After pouring off the acetic acid-ethyl alcohol, 70% ethyl alcohol was added and remained on the cells for at least 30 min. The cover slips were then put on a Chen type B staining rack (Arthur H. Thomas Co., Philadelphia, Pa.) and placed in ice-cold 0.5 M perchloric acid or 5% trichloroacetic acid for 20 to 30 min. They were washed two times with distilled water and were dipped briefly in absolute ethyl alcohol-ether (1:1) and then in ether. After the ether had evaporated, the cover slips were placed in counting vials, a toluene base scintillation fluid was added, and the samples were counted in a Mark I Nuclear-Chicago scintillation counter.

Chemicals. Trypsin (1:250) was obtained from Difco. It was routinely used at a concentration of 0.25% in removing a monolayer of chick embryo cells from the surface of a vessel. Sterile ethylenediaminetetraacetic acid (EDTA) solution (1:5,000) was obtained from Grand Island Biological Co. Hydroxyurea was a gift from E. R. Squibb and Sons, Inc., New Brunswick, N.J. Powdered Eagle MEM, fetal calf serum, and gamma globulin-free fetal calf serum were purchased from Grand Island Biological Co.

RESULTS

Action of interferon in cells at different times after seeding. Secondary chick embryo cells were seeded in petri dishes (2 × 10^6 cells per dish) and received either MEM or 6 units of interferon 17, 41, or 65 hr later. After a 4-hr incubation period at 36 C, the cells were challenged with VSV at a multiplicity of 3 PFU/cell and a yield experiment was carried out. Table 1 shows that the cells became more sensitive to the action of interferon the longer they remained in culture. This "aging" effect occurred even though the cells had not reached confluency by 65 hr.

TABLE 1. *Action of interferon in cells at different times after seeding*

Time after seeding	Cells/dish[a]	Yield of VSV (PFU/cell)		
		Control	Interferon	Per cent control
hr				
17	1.3 × 10^6	64	3.96	6.2
41	2.7 × 10^6	64	1.48	2.3
65	3.2 × 10^6	80	0.42	0.52

[a] Estimated by cell count on duplicate cultures at the time of addition of interferon.

Interferon action and DNA synthesis at different times after seeding. Secondary chick embryo cells were found to attach to the bottom of a petri dish by 3.5 hr after seeding, enabling us to include very young cells in these experiments. Interferon action was studied as above, except that 12.5 units of interferon was used. DNA synthesis was measured as described above.

The 66-hr-old cultures were fully confluent, as can be seen by the high cell count and by the very low rate of DNA synthesis (Table 2). (The differences in cell densities in the results shown in Tables 1 and 2 can probably be explained in terms of variations in plating efficiency.) Both the 5- and 17-hr-old cells synthesized DNA at a high rate and both were less sensitive to interferon action than the 66-hr-old cells.

Effect of hydroxyurea on interferon action. Hydroxyurea is able to inhibit DNA synthesis, probably by inhibiting ribonucleoside diphosphate reductase (15, 26). Secondary chick embryo cells were preincubated for 1 hr in 1,250 μg of hydroxyurea per ml or in MEM before receiving either 2 units of interferon or MEM. The hydroxyurea-treated cells were also in contact with the drug during the subsequent incubation with interferon or MEM. The cells had been seeded at a slightly lower density (2 × 10^6 cells per dish) so that complete confluency would not be obtained until several days after seeding.

Table 3 shows that, at both 23 and 70 hr, the cell counts were low and the rate of DNA synthesis was high, indicating that in neither case had the cells been confluent. The growth rate (as indicated by the rate of DNA synthesis) was somewhat lower in the 70-hr-old cultures.

The hydroxyurea inhibited at least 75% of the DNA synthesis, but the efficiency of interferon action was not affected by the drug. There was an increase in the yield of VSV from the hydroxyurea-treated cells (in both control and interferon-

TABLE 2. *Interferon action and DNA synthesis at different times after seeding*

Time after seeding	Cells/dish[a]	Yield of VSV (PFU/cell)			^{3}H-thymidine incorporation	
		Control	Interferon	Per cent control	Counts per min per cover slip	Normalized counts per min per cover slip[b]
hr						
5	2.2×10^6	70	0.18	0.26	1,597	725
17	3.7×10^6	96	0.16	0.17	2,980	780
66	5.7×10^6	134	0.059	0.044	236	41

[a] See Table 1.
[b] Counts per min per cover slip/cell count $\times 10^6$.

TABLE 3. *Effect of hydroxyurea on interferon action*

Time after seeding	Cells/dish[a]	Treatment	Yield of VSV (PFU/cell)			^{3}H-thymidine incorporation (normalized counts per min per cover slip)[b]
			Control	Interferon	Per cent control	
hr						
23	0.96×10^6	None	46.0	2.68	5.8	1,640
		Hydroxyurea[c]	54.3	3.28	6.1	406
70	1.94×10^6	None	47.0	0.268	0.57	1,345
		Hydroxyurea[c]	75.8	0.433	0.57	158

[a] See Table 1.
[b] See Table 2.
[c] Incubated with 1,250 μg of hydroxyurea per ml for 1 hr before, and during, treatment with interferon.

treated cells). This may reflect the greater availability of ribonucleotide diphosphates in these cells.

Action of interferon in secondary cells obtained from trypsinized EDTA-treated cultures. It is conceivable that the low efficiency of interferon action in young cells is due to trypsin digestion of receptor sites for interferon. EDTA is a chelating agent which removes cells from a surface by removing the ions needed for attachment and is therefore less likely to cause permanent destruction to receptor sites. When a solution of 0.25% trypsin was added to a monolayer of chick embryo cells, the cells came off the surface of the flask and also separated from each other to form a uniform suspension. But when an EDTA solution (1:5,000) was added, the cells came off the flask in one continuous sheet. Rapid pipetting was necessary to break apart the clumps of cells. Trypsinized cells in the experiment were treated in a similar manner as the control. Even after rapid pipetting, the EDTA-treated cells contained some clumps, making cell counts unreliable.

Either MEM or 6.4 units of interferon was in contact with the cells for 3 hr before challenge with VSV. Table 4 shows that both trypsinized and EDTA-treated cells became more sensitive to interferon action with age. The increase in sensitivity is quite similar in both groups of cells.

TABLE 4. *Action of interferon in secondary cells obtained from trypsinized and EDTA-treated cultures*

Group	Time after seeding	Yield of VSV (PFU/culture)		
		Control	Interferon	Per cent control
	hr			
Trypsin	4	2.4×10^7	2.6×10^6	10.8
	23	5.1×10^7	8.4×10^5	1.6
EDTA	4	3.0×10^7	2.2×10^6	7.3
	23	5.4×10^7	4.3×10^5	0.8

Interferon action and cell density. To test the effect of cell density on interferon action, petri plates were seeded with 0.75, 1.5, or 3.0 $\times 10^6$ secondary chick embryo cells. At 17 and 41 hr after seeding, the cells were treated for 4 hr with 4 units of interferon or MEM, after which a yield experiment was carried out (Table 5). It is clear that an increase in cell density resulted in increased sensitivity to the action of interferon. Within any one group seeded at a certain density,

TABLE 5. *Interferon action in secondary cells seeded at different concentrations*

No. of cells seeded per petri dish	Time after seeding	Yield of VSV (PFU/culture)		
		Control	Interferon	Per cent control
	hr			
3×10^6	17	2.0×10^7	10^6	5.0
	41	5.2×10^7	8.8×10^5	1.7
1.5×10^6	17	4.4×10^6	6.8×10^5	15.4
	41	1.1×10^7	6.4×10^5	5.8
0.75×10^6	17	2.3×10^6	4.4×10^5	19.1
	41	3.2×10^6	5.2×10^5	16.2

the sensitivity of cells to interferon action was increased with their time in culture.

DISCUSSION

Carver and Marcus (3) postulated that some time-dependent process alters cultured cells so that they become more susceptible to interferon action. But they felt that this aging process may be initiated only after a critical cell density is reached. In their work, even after the cells were fully confluent, further aging increased their sensitivity to interferon action. Because this was true for interferon production as well as for interferon action, it was suggested that the aging process may represent a general phenomenon common to all inducible processes.

Our data show that, even before the cells became confluent, the amount of time in culture influenced the sensitivity of cells to interferon action. Therefore, it seems unlikely that a critical density is necessary for the aging process. On the other hand, cell density is an important factor in determining the sensitivity of cells to interferon action. It may even be a major component of this phenomenon.

As cells become confluent, there is an overall decrease in the rate of macromolecular synthesis (11). Increasing the rate of protein synthesis by various methods has been shown to result in a decrease in the production of interferon (5). Previously we reported a decrease in sensitivity to interferon in cells treated with serum, which is able to stimulate cellular RNA and protein synthesis (25). It has been suggested (5) that cells synthesizing protein at a high rate also synthesize more of a repressor and are therefore more difficult to induce. Young, nonconfluent cell cultures (according to this argument) are less sensitive to interferon action because they synthesize protein (repressor) at a higher rate than older confluent monolayers.

The phenomenon of increased sensitivity to interferon action in older cells shows some resemblance to observations in the rat liver (17, 18). In the mitotically inactive adult liver, the levels of tryptophan pyrrolase synthesized in response to hydrocortisone are much higher than in the dividing cells of the regenerating liver. More specifically, it is during the period after partial hepatectomy when DNA synthesis is known to occur that the loss of inducibility is the greatest. It was thus suggested that there may be an incompatibility between DNA synthesis and the transcription of messenger RNA. Our data cannot be explained on this basis. The inhibition of DNA synthesis in growing cells did not increase the sensitivity of the cells to interferon action. Moreover, other workers have shown that RNA synthesis is inhibited only where the chromosomes are condensed during replication, but that RNA continues to be synthesized in the nonreplicating regions (16, 20). On the other hand, it seems quite reasonable to assume that induction should be inhibited during mitosis, since there is little or no RNA synthesis in animal cells during that time (6, 9, 10, 16, 22). In fact, colchicine has been found to inhibit both the production and the action of interferon (19). Although the increased sensitivity of aged cells cannot be explained solely in terms of a decreased mitotic rate, it is conceivable that this is a contributing factor.

Out data also show that the decreased sensitivity in newly seeded cells is not the result of trypsin digestion of receptor sites for interferon. Cantell and Paucker (2) also reported that removal of the monolayer of HeLa cells by scraping or by EDTA gives similar results in aging experiments. Our finding that the sensitivity of cells to interferon action varies with the number of cells seeded per petri dish also indicates that destruction of receptor sites is not a likely explanation for the decreased sensitivity of newly seeded cells.

Thus, although the reasons for the increase in sensitivity to the action of interferon with the "aging" of chick embryo cells in vitro could not be fully explained, two factors were ruled out as the underlying cause of this phenomenon. They are (i) the rate of cellular DNA synthesis and (ii) the regeneration of a cell receptor for interferon with aging. Cell density was definitely found to play a role, but since the growth rate decreases with increased cell density and since RNA synthesis is inhibited during mitosis, it is difficult to separate effects resulting from increased density from effects resulting from a decreased mitotic rate.

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