INDUCTION OF THE DIVISION CYCLE IN RESTING STAGE HUMAN FIBROBLASTS AFTER RSV INFECTION

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Several reports have appeared (Winocour et al., 1965, Dulbecco et al., 1965 and Weil et al., 1965) showing that deoxyribonucleic acid (DNA) containing viruses were able to induce DNA synthesis in monolayers where the cell division cycle had stopped due to crowding (cell cycle inhibition). It has not been described so far if ribonucleic acid (RNA) containing viruses are capable of producing the same effect. Rous sarcoma virus (RSV) is a RNA virus which requires DNA synthesis during the early stages of the infectious cycle (Bader, 1964 and 1965; and Temin, 1964). For this reason we decided to see if RSV is also capable of releasing a monolayer from cell cycle inhibition. Results show that RSV infection induces DNA synthesis and mitoses in cell cycle inhibited cultures and that the intact virus particle may not be identical with the stimulating principle.

Materials and Methods. A line (HEB) of human embryonic fibroblasts was used in these studies, maintained in Eagle's minimal essential medium (Eagle, 1959) supplemented with 10% calf serum, penicillin (100 U/ml), streptomycin (50 μg/ml) and aureomycin or kanamycin (50 μg/ml), as described elsewhere (Macieira-Coelho et al., 1966). The Schmidt-Ruppin (RSV-SR) and the Engelbreth-Holm (RSV-EH) strains of RSV (Stenkvist et al., 1964) were used to infect monolayers in resting stage. The titer of the RSV-EH pool used was 10^6 focus forming units (FFU)/ml measured on chicken embryo fibroblasts. Four to five days after subcultivation when only mi-

nimal mitotic activity could be seen, medium was removed and the monolayers exposed to 0.1 ml of undiluted virus for 1 hour at 37°C. After this period medium was again added together with tritium labeled thymidine (H3-TdR) with a specific activity of 1.9 C/mM and in a final concentration of 0.01 μC/ml. Mock infected cultures were treated exactly the same way, only 0.1 ml of serum-free medium was added instead of virus suspension. Control cultures were left untouched, only H3-TdR was added at the same time as to infected cultures. Twenty-four hours after infection the cultures were washed with phosphate balanced salt solution, fixed in acetic acid:methanol (1:3) and processed for autoradiography with Kodak-AR10 film. The % labeled interphases was determined from the analysis of 500 cells and mitotic indices from the analysis of 3000 cells.

Virus neutralization was done the following way: RSV-SR anti-serum prepared in chickens and control serum (both obtained from Dr. Jonsson, University of Lund, Sweden) were heated at 56°C for 30 minutes, diluted 1:2 with Eagle's medium, added 1:1 to the virus suspension and kept for 2 hours at 37°C. These suspensions (virus treated with anti-serum and with control serum) were then immediately used to infect the cultures.

Experimental an Results. Cultures were pooled and distributed evenly into new Petri dishes. Four days later, when only occasional mitoses could be seen, duplicate cultures were infected with RSV-SR or RSV-EH, new medium was added with H3-TdR and the cultures examined for % labeled interphases and mitotic indices (Table I). In both experiments, while the amount of cells synthesizing DNA and the mitotic indices in the controls were negligible, these two parameters increased significantly in the virus infected cells. Mock-infected cells however, also showed a significant increase of DNA-synthesizing cells and mitoses, although less than the increase in RSV-infected cultures. Fourty-eight hours after infection mitotic activity had disappeared and the cultures were again in resting stage. Since Todaro et al. (1965) have shown that fresh serum is capable of releasing cells from cell cycle inhibition, we repeated the mock

TABLE I

% labeled interphases and mitotic indices 24 hours after infection of resting stage cultures with RSV-SR or RSV-EH.

	RSV-SR		RSV-EH	
	% labeled interphases	mitotic indices	% labeled interphases	mitotic indices
controls	9	0.3	12	0.2
mock infected	28	1.2	41	2.0
infected	41	2.6	56	6.5

infection using fresh medium in one group of cultures and spent medium (medium which had been in contact with the cells from subcultivation to resting stage) to another group (Table II). It is clear that mock

TABLE II

% labeled interphases and mitotic indices 24 hours after mock infection of resting stage cultures. One group received fresh medium, the other received medium which had been in contact with the cultures from the time of subcultivation to the time the experiment was performed.

	% labeled interphases	mitotic indices
controls	12	0.3
mock infected spent medium added	10	0.6
mock infected fresh medium added	34	2.9

infection per se did not release cells from cell cycle inhibition and that the effect obtained in the first experiments with mock in-

fection was due to the medium change. For this reason, spent medium was always added after infection in subsequent experiments.

To see if the stimulatory effect obtained with the virus infection could be suppressed by anti-serum, cultures were infected with virus suspension treated with anti-serum and control serum. Table III shows that a 4-fold increase was obtained in all the infec-

TABLE III

Mitotic indices 24 hours after infection of resting stage cultures with RSV-SR suspensions and with the same suspensions treated with anti-serum and control serum.

infected with	mitotic indices
-	0.7
RSV-SR	2.8
RSV-SR anti-serum	2.5
RSV-SR control serum	2.2

ted groups as compared with the controls, regardless of the presence of anti-serum. The same anti-serum was checked for the capacity to inhibit focus formation in chicken embryo fibroblasts. As shown in Table IV the anti-serum inhibited the focus formation completely, while the control serum did not.

RSV-SR suspensions were incubated at 37°C for 30 minutes with 100 µg/ml deoxyribonuclease (DNAase), 100 µg/ml ribonuclease (RNAase) and 1 mg/ml crystaline trypsin. The trypsin mixture was then treated with 1 mg/ml trypsin inhibitor for 10 minutes at room temperature. Resting stage cultures were infected with RSV-SR and with RSV-SR treated with the different enzymes, the cultures were fixed 24 hours later and the % labeled interphases counted after autoradiography.

TABLE IV

Titer of RSV-SR suspensions and of the same suspensions treated with control serum and anti-serum.

infected with	FFU/ml
RSV-SR	10^4
RSV-SR treated with control serum	10^4
RSV-SR treated with anti-serum	0

TABLE V

% labeled interphases 24 hours after infection of resting stage cultures with RSV-SR and with RSV-SR treated with different enzymes.

infected with		% labeled interphases
RSV-SR		27
RSV-SR treated with	RNAase	30
	DNAase	15
	trypsin	19
	-	16

As can be seen in Table V, the stimulatory activity present in RSV-SR suspensions could be abolished with DNAase and trypsin but not with RNAase.

Discussion. Results show that suspensions containing RSV-SR and RSV-EH are capable of releasing human fibroblasts from the inhibition of the cell cycle normally present once the cell number has reached a certain critical value. When spent medium was employed the reaction could be shown to be specifically associated with the

RSV containing suspensions. The component in the suspension responsible for the stimulation of DNA synthesis and mitosis, has not been identified. The present results indicate that the intact virus particle may not be identical with the stimulating principle. Anti-serum with a strong inhibitory effect on the focus forming capacity of the RSV-SR suspension on chicken fibroblasts, failed to decrease the stimulatory effect on the cell cycle of the same suspension. Also DNAase and trypsin destroyed the activity of the stimulatory principle, while RNAase did not. Although it is tempting to suggest that the DNA stimulatory effect may be connected with the cell alterations which eventually occur in cultures of human fibroblasts exposed to RSV (Stenkvist et al., 1964), it should be stressed that the stimulatory effect was seen within 24 hours whereas morphologic signs of alteration cannot be detected until at least 2 weeks postinfection (Stenkvist et al. 1964). Consequently there is no definitive evidence that stimulation of DNA synthesis is causally related to cell alteration.

Data obtained with Rauscher virus (to be published) have shown that suspensions containing the latter (another RNA virus) are also capable of inducing division in resting stage human fibroblastic cultures.

References

Bader, J. P., Virology 22, 462 (1964).
Bader, J. P., Virology 26, 253 (1965).
Dulbecco, R., Hartwell, L. H. and Vogt, M., Proc. Nat. Acad. Sc. 53, 403 (1965).
Eagle, H., Science 130, 432 (1959).
Macieira-Coelho, A., Pontén, J. and Philipson, L., Exp. Cell Res. 43, 20 (1966).
Stenkvist, B. and Pontén, J., Acta Path. Microb. Scand. 62, 315 (1964).
Temin, H. M., Virology 23, 486 (1964).
Todaro, G. J., Lazar, G. K. and Green, H. J., J. Cell. Comp. Phys. 66, 325 (1965).
Weil, R., Michel, M. and Rushman, G., Proc. Nat. Acad. Sc. 53, 1468 (1965).
Winocour, E., Kaye, A. M. and Stollar, V., Virology 27, 156 (1965).