The Incorporation of Thymidine into Deoxyribonucleic Acid in Mouse Fibroblasts Infected with Polyoma Virus

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1. Mouse-fibroblast cultures in the stationary phase of growth show an increased rate of [^3H]thymidine incorporation into DNA from 12 to 44 hr. after infection with polyoma virus. 2. Intracellular virus progeny is first detected at about 24 hr. after infection. 3. Calculations based on the [^3H]thymidine-incorporation data and direct measurements of the DNA content of the cell cultures indicate that the amount of the excess of DNA synthesized by the infected cell cultures corresponds to about 10% of their total DNA. 4. The mitotic index of the cell cultures at 40 hr. after infection was significantly higher than that of non-infected control cells. 5. Possible interpretations of the stimulation of DNA synthesis observed in polyoma-infected cell cultures are discussed.

Studies on a number of animal cells infected with various DNA viruses have shown that, soon after infection, marked changes occur in the normal pattern of DNA synthesis of the cell (Ben-Porat & Kaplan, 1963; Green & Daesch, 1961; Ginsberg & Dixon, 1959; Newton & Stoker, 1958).

The present work was undertaken to study the effect of polyoma infection on the process of DNA synthesis in mouse-fibroblast cultures. It was found that in the stationary phase of growth these cell cultures show an increased rate of incorporation of [^3H]thymidine into DNA 12 hr. after virus adsorption. Direct measurements of the total DNA content of the cultures showed that the enhanced rate of thymidine incorporation corresponds to an accumulation of DNA in the infected cultures that greatly exceeds the amount required for virus production.

Increased DNA synthesis in polyoma-infected cells has been observed previously by Minowada & Moore (1963) by radioautography, and by Dulbecco, Hartwell & Vogt (1965), who showed, in addition, that most of the newly synthesized DNA was host DNA.

In the present paper we report the results of experiments that confirm the previous observations and give additional data on some aspects of the problem of the determination of the amount of DNA synthesized by the infected cell cultures, the stimulatory effect on DNA synthesis of the addition of fresh medium to the cell cultures and the mitotic index of the cell cultures at different times after infection.

MATERIALS AND METHODS

Secondary cultures of mouse-embryo cells were prepared by seeding 2×10^6 cells in 100 mm. Falcon plastic Petri dishes with 10 ml. of reinforced Eagle medium (fourfold concentration of amino acid and vitamins) supplemented with 10% of calf serum (Smith, Freeman, Vogt & Dulbecco, 1960). Cell counts were performed in a Bürker chamber. The cells were incubated under a flow of humidified CO_2 -air mixture for 48 hr. After that time the medium was replaced with medium containing 10% of horse serum; 48 hr. later the medium was removed and the cell cultures were infected by the addition of 0.25 ml. of an S.E. polyoma suspension (originally obtained from Dr B. Eddy), containing 4×10^8 plaque-forming units/ml. (30 plaque-forming units/cell). The cells were incubated for 3 hr., and then the inoculum was decanted and the same medium present before infection was again added to the cultures (see the Results section).

Virus titration was carried out, after the cells had been disrupted by an ultrasonic treatment, by plaque assay as described by Winocour & Sachs (1960).

For the thymidine-incorporation experiments, $5 \mu\text{C}$ of [^3H]thymidine, of specific activity 4C/m-mole (The Radiochemical Centre, Amersham, Bucks.), was added to the cultures. Incubation was stopped after 1 hr. by adding 0.3 ml. of aq. HClO_4 (final concn. 0.2 N). Subsequent operations were performed at $2-4^\circ$. The cell residues were scraped off with a rubber 'policeman', transferred to a centrifuge tube and spun for 5 min. at 2000g. The sediment was washed twice with 0.2 N- HClO_4 and then once in ethanol and once with ethanol-ether (3:1, v/v). Finally, the sediment was suspended in 2 ml. of 0.6 N- HClO_4 and heated at 70° for 20 min. On a portion of this hydrolysate the DNA content of the cell cultures was determined by using the diphenylamine method of Burton (1956). The ^3H -radioactivity was determined on another portion of the same

solution by using a Tracerlab liquid-scintillation counter, model LSC-20, an appropriate internal standard being used to allow for correction of the quenching caused by the acid solution. The efficiency of scintillation counting was 5%.

The mitotic index of the cultures was determined by counting all mitotic figures on seven or eight different cell preparations. Groups of 1000 cells were examined for each cell preparation. Bouin's fixative and haematoxylin-eosin staining were used.

RESULTS

Growth pattern of mouse-fibroblast cultures. The number of cells adherent to the plastic surface at 24 hr. after seeding was about 1×10^6 and increased up to 4×10^6 by 72 hr., then slightly decreased.

The DNA content of the cultures increased linearly from 24 to 72 hr., whereas the specific ^3H -radioactivity of DNA decreased and then remained practically constant at very low levels (Fig. 1). The rapid fall of the curve of the ability to incorporate thymidine at the time when DNA synthesis is still going on is for the greater part the result of the increasing DNA content of the cultures. The low levels of the second part of the curve of the ability to incorporate thymidine is in accord with the extremely limited DNA synthesis occurring in the cultures after 72 hr. The experiment reported in Fig. 1 shows that, from 24 to 48 hr. after plating, the DNA content of the cultures

increases by about $18 \mu\text{g}$. On the basis of the thymidine-incorporation data, it was calculated that, during the same period, only $0.27 \mu\text{g}$. of ^3H -thymidine can be incorporated into DNA. As this amount of thymidine corresponds to that contained in $1.3 \mu\text{g}$. of DNA, it appears that the coefficient of utilization of the exogenous ^3H -thymidine for DNA synthesis in our system is only 7.3%. This low thymidine utilization is not due to a lack of precursor, as during the experiment large amounts of ^3H -thymidine remain in the medium. Since it is known that, in mammalian cells, most of the DNA thymine is derived from the endogenous deoxyuridylic acid (Humphreys & Greenberg, 1958), this result is not surprising.

Effect of the addition of fresh medium to the cell cultures. During the present experiments it was noted that the simple replacement of the medium of the cell cultures with new medium, as is generally carried out after virus infection, produced a marked stimulation of the incorporation of ^3H -thymidine into DNA for a period of a few hours.

This effect is shown in the experiment reported in Fig. 2, in which the rate of incorporation of ^3H -thymidine into DNA in cell cultures at different times after plating was measured. The medium was not changed in the cultures for 140 hr., and then at 140 and 240 hr. the medium was replaced with fresh medium.

Fig. 1. Incorporation of ^3H -thymidine into DNA ($\circ$) and the total DNA content (Δ) in mouse-fibroblast cultures as a function of the cell age. Experimental details are given in the text.

Fig. 2. Effect of replacing the medium in mouse-fibroblast cultures in the stationary phase of growth. $\circ$, Specific ^3H -radioactivity of DNA of cell cultures labelled with ^3H -thymidine at the times noted; at 140 and 240 hr. after plating the medium of the cell cultures was replaced with fresh medium. Δ , Specific ^3H -radioactivity of DNA of cell cultures in which at 140 and 240 hr. the medium was replaced with medium obtained from 72 hr.-old cultures. Experimental details are given in the text.

At 24 hr. after the replacement of the medium, the rate of [^3H]thymidine incorporation into DNA was significantly increased. On the other hand, if the replacing medium was obtained from a 72 hr. or older culture, no rise of the thymidine-incorporation rate occurred. The reason for the stimulating effect of fresh medium was not investigated further.

Experiments with polyoma-infected cells. On the basis of the results reported above, it was decided to replace the medium of the cultures 48 hr. after plating and to infect the cells 48 hr. later, when the rate of [^3H]thymidine incorporation is again low. After the virus was adsorbed, the same medium present in the cultures before infection was again added to the cell instead of fresh medium.

Control samples were handled in exactly the same way except that a non-infectious cell lysate was added instead of virus. The results of one experiment are shown in Fig. 3. In comparison with the control samples, the infected cell cultures show a marked increase in the rate of thymidine incorporation into DNA between 12 and 44 hr. after infection.

Assuming that the coefficient of utilization of the exogenous thymidine in this cell system does not change as a consequence of the virus infection, it was calculated that, between 12 and 44 hr. after the infection, about 3.3 μg . of extra DNA was synthesized in each infected culture. These results were confirmed by direct determinations of the

DNA content of the cell cultures in a number of samples sufficiently large to permit the statistical evaluation of the results (Table 1). At 24 hr. after infection, the DNA content of infected cultures was about 4 μg . greater than that of normal cultures. No further increase in the DNA content of the infected cell cultures was detected at 44 hr. after infection. However, from 26 or 28 hr. after infection some cells start to release virus particles and

Fig. 3. Incorporation of [^3H]thymidine into DNA in mouse fibroblasts infected with polyoma. $\circ$, Control cells; $\bullet$, infected cells; Δ , virus titre.

Table 1. *DNA contents of normal and polyoma-infected mouse-fibroblast cultures*

Experimental details are given in the text. The DNA contents are given as means $\pm$ S.D.

Time after infection (hr.)		No. of determinations	DNA content ($\mu\text{g.}/\text{culture}$)	Significance of difference (Student's t test)
24	Normal cultures	20	42.79 ± 4.06	$t = 3.42$ $0.01 > P > 0.001$
	Infected cultures	20	46.20 ± 3.09	
44	Normal cultures	20	43.37 ± 3.05	$t = 4.38$ $0.01 > P > 0.001$
	Infected cultures	20	47.50 ± 2.90	

Table 2. *Mitotic indexes of normal and polyoma-infected mouse-fibroblast cultures*

Experimental details are given in the text; 1000 cells were counted for each determination. The mitotic indexes are given as means $\pm$ S.D.

Time after infection (hr.)		No. of determinations	Mitotic index	Significance of difference (Student's t test)
20	Control cultures	7	0.60 ± 0.200	$t = 2.26$ $0.05 > P > 0.02$
	Infected cultures	7	0.40 ± 0.129	
40	Control cultures	8	0.52 ± 0.138	$t = 5.133$ $0.01 > P > 0.001$
	Infected cultures	8	0.81 ± 0.180	

to lyse, and it is possible that after that time a part of the DNA present in these cells is degraded.

Intracellular virus started to form, as judged from the plaque assay, at about 24hr. after the infection.

Mitotic index of the cell cultures. The results of this study are shown in Table 2. It appears that at 20hr. after infection the mitotic index of the infected cultures was slightly lower than that of the control cultures. On the other hand, at 40hr. after infection the mitotic index of the infected cell cultures was significantly higher than that of the control cells.

DISCUSSION

The present results indicate that mouse-fibroblast cultures in the stationary phase of growth show a marked increase in the rate of DNA synthesis from 12 to 44hr. after infection with polyoma virus. Calculations based on the results of experiments on the incorporation of [³H]thymidine into DNA suggest that about 3.3 μ g. excess of DNA is synthesized in the infected cultures.

As the virus may have altered the permeability of the cell membrane in some way, the results of experiments based on the incorporation of exogenous precursors may not necessarily be meaningful. It was therefore important to confirm by direct measurements that there was a real increase of the DNA content in the infected cultures. The results of these experiments indicated that the excess of DNA present in the infected cultures was about 4 μ g., corresponding to 10% of their total DNA. From the data published by Crawford, Crawford & Watson (1962) on the DNA content of the virus particles and the efficiency of the plaque assay, the total amount of virus found in our experiments 48hr. after infection should not contain more than 0.01–0.02 μ g. of DNA.

Minowada & Moore (1963) by radioautographic experiments, and Dulbecco *et al.* (1965) by experiments on the incorporation of [³H]thymidine and [³²P]phosphate into DNA, previously reported the occurrence of a stimulation of DNA synthesis in mouse-embryo cells and mouse-kidney cells infected with polyoma virus. Minowada & Moore (1963) concluded that this effect was related primarily to virus production. Dulbecco *et al.* (1965), on the other hand, showed that the labelled precursors were incorporated predominantly into cellular DNA and only for a minor part into viral DNA. These

authors suggested that the virus may stimulate the synthesis of cellular DNA in the infected cell with a mechanism probably similar to that involved in the process of cell transformation.

This attractive hypothesis, however, does not convincingly account for the present results. It is known, in fact, that in our experimental conditions not more than 5% of the cells of the cultures are initially infected and produce the virus. The remaining cells are either infected abortively or are not infected at all. If the increase of DNA observed were due to the infected cells, we should expect to find in these cells a 20-fold increase of their DNA. Previous cytological experiments, however, failed to show any large increase of DNA in polyoma-infected cells (Leuchtenberger, Leuchtenberger, Stewart & Eddy, 1961).

It seems more probable that in our cell cultures the increased DNA synthesis observed was due to the non-infected cells or to the abortively infected cells, which were stimulated in some indirect way to synthesize DNA and to divide.

Further studies at cytological levels are required to decide which type of cell is really responsible for the increased DNA synthesis observed in polyoma-infected cell cultures.

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REFERENCES

- Ben-Porat, T. & Kaplan, A. S. (1963). *Virology*, **20**, 310.
- Burton, K. (1956). *Biochem. J.* **62**, 315.
- Crawford, L. V., Crawford, E. M. & Watson, D. H. (1962). *Virology*, **18**, 170.
- Dulbecco, R., Hartwell, L. H. & Vogt, M. (1965). *Proc. nat. Acad. Sci., Wash.*, **53**, 403.
- Ginsberg, H. S. & Dixon, M. K. (1959). *J. exp. Med.* **109**, 407.
- Green, M. & Daesch, G. E. (1961). *Virology*, **13**, 169.
- Humphreys, G. K. & Greenberg, D. M. (1958). *Arch. Biochem. Biophys.* **78**, 275.
- Leuchtenberger, R., Leuchtenberger, C., Stewart, S. E. & Eddy, B. E. (1961). *Cancer*, **14**, 567.
- Minowada, J. & Moore, G. E. (1963). *Gann*, **54**, 59.
- Newton, A. & Stoker, M. G. P. (1958). *Virology*, **5**, 549.
- Smith, J. D., Freeman, G., Vogt, M. & Dulbecco, R. (1960). *Virology*, **12**, 185.
- Winocour, E. & Sachs, L. (1960). *Virology*, **13**, 699.