A STUDY OF THE MECHANISMS OF DNA AND THYMINE DEGRADATION IN CULTURED HUMAN CELLS INFECTED WITH A LIPOVIRUS*

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The preceding articles describe the biological, immunological, and physico-chemical properties of a lipovirus, and the release of a lipogenic toxin from infected cells (1, 2). This report describes experiments designed for the elucidation of the mechanisms of DNA and thymine degradation in cultured cells infected with this lipovirus.

Materials and Method

Cell Cultures.—Cultivation of the "liver" and other established cells was similar to that described in the preceding report (1). Primary human amnion (obtained from full term placentae), primary human (from embryos 3 to 5 months of age), chick, and mouse embryonic tissues were prepared by the trypsin dispersion method and grown on glass as "monolayer."

Thymidine-2-C^{14}-Labeled Cells.—C^{14}-labeled cultures were prepared by growing the cultures in nutrient medium containing 0.5 μc thymidine-2-C^{14} per ml for 48 to 96 hours, rinsed 3 times with 2 ml of regular medium (without C^{14} substrate), and allowed to grow for another 24 to 48 hours in the regular medium. Primary amnion cells were frequently cultured for longer duration in thymidine-2-C^{14} medium to increase incorporation of radioactivity. Two lots of thymidine-2-C^{14} with specific activity of 6.25 and 1.43 mc per mmole, purchased from the New England Nuclear Corporation, Boston, were used in these experiments.

Fractionation of C^{14}-Labeled Cultures.—Based on the results described in an earlier report (3), each culture was fractionated into CO_2, acid-soluble, and acid-insoluble fractions (the last fraction contained all the DNA). Used media from cultures which failed to show any cell destruction within 3 to 4 days after infection were kept frozen and were added to their corresponding cultures at the time of fractionation. The fractionation procedure was as follows: (*a*) 0.1 of 100 per cent trichloroacetic acid was added to the culture which contained 1 ml of medium and was placed in an ice bath; (*b*) an alkaline trap was quickly installed and kept for 1 hour to absorb the CO_2 (4); (*c*) used media or 1 ml of fresh medium (for cultures destroyed within 3 to 4 days) together with $\frac{1}{10}$ volume of 100 per cent trichloroacetic acid were added; (*d*) after another hour, the mixture was centrifuged at 3000 RPM for 15 minutes in a refrigerated centrifuge; (*e*) the acid-soluble fraction was drained off as completely as possible, and (*f*) the acid-insoluble fraction was hydrolyzed in its original volume of 0.1 N NaOH without further washing (since less than 5 to 10 per cent of the acid-soluble fraction was estimated to be trapped in the acid-insoluble material). In experiments where more accurate measurement of the radioactivity of the acid-insoluble fraction was required, the fraction was washed 3 times with 5 ml of 10 per cent cold trichloroacetic acid prior to hydrolysis with NaOH.

Measurement of C^{14} Activity.—The alkaline trap was dissolved in 2 ml H_2O, half of which was monitored for C^{14} activity. Since this direct plating without the precipitation of CO_2 as $BaCO_3$ reduced the radioactivity by 67.7 per cent (average of 12 determinations), the actual count registered for each CO_2 sample was multiplied by 3.1. The acid-soluble fraction was extracted 2 times with twice its volume of ether to remove most of the trichloroacetic acid prior to monitoring of C^{14} activity. The amount of interference introduced by the precipitates

TABLE I

DNA and Thymine Degradation in Thymidine-2-C^{14}-Labeled "Liver" Cells Infected with the Lipovirus

Exp.	Day		Radioactivity* in cell fractions	
			Infected	Uninfected
1	0			(0) 0/210/6038
	1	(0)‡	0/88/6038§	(0) 0/126/6430
	4	(+)	348/582/6038	
	6	(+++)	3089/2461/556	(0) 0/276/5978
2	0			(0) 0/38/7050
	5	(+)	99/333/5240	(0) 0/224/5620
	7	(+++)	3660/1560/1950	(0) 0/190/4560
		(+++)	2480/1310/1630	(0) 0/253/6050
	14	(+++)	3450/2390/1320	(0) 0/342/6440
		(+++)	2480/2130/965	(0) 0/182/3070
3‖	0			(0) 0/53/13240
	7	(+++)	3521/6212/612	(0) 0/736/14316
	14	(+++)	6119/3439/252	

* Expressed as counts per minute above background per culture.

‡ (0), (+), (++), (+++) signified no, early, advanced, and complete degeneration.

§ CO_2/acid-soluble/acid-insoluble.

‖ Acid-insoluble fractions washed 3 times before hydrolysis.

in both the acid-soluble and acid-insoluble fractions were determined by plating similar fractions obtained from unlabeled culture on planchets with C^{14} activity of about 500 counts/min. Radioactivity was found reduced by 47.4 per cent and 44.5 per cent for acid-soluble and acid-insoluble fractions, respectively. Correction factors of 1.9 and 1.8 were therefore used. All fractions were monitored as thin specimen with a gas flow Geiger-Müller tube. Total time required to register 1000 counts was determined. The results were expressed as counts per minute above background per culture. Samples which registered less than 3 counts/min above background (11 to 14 counts/min) were arbitrarily considered negative.

Viruses and Rickettsia.—Cell-free form of the lipovirus prepared as described (1) was used. Herpes simplex, adeno 3, polio 1 (Brunhilde strain), polio 2, and Coxsackie B1 viruses were strains used routinely in this Laboratory; they have undergone unknown numbers of passages through several established strains of human cells. Vaccinia virus was harvested from the

chorioallantoic membrane of chick embryos. Sindbis virus was kindly furnished by Dr. M. Ho of the University of Pittsburgh; Sandai virus by Dr. I. Graser of the Children's Hospital in Boston; encephalomyocarditis virus and Rous sarcoma virus, by Dr. M. D. Eaton; polyoma virus by Dr. J. Levinthal; mouse brain passage rabies and Theiler's virus, from Dr. E. S. Murray of this Department.

Degradation Study.—A replicate set of labeled cultures was used in each experiment. 2

TABLE II

DNA and Thymine Degradation in Various Thymidine-2-C¹⁴-Labeled Cells Infected with the Lipovirus

Cell	Day		Radioactivity* in cell fractions	
			Infected	Uninfected
Human amnion	12	(++)‡	239/174/65§	(0) 0/53/724
	16	(+++)	326/278/124	(0) 0/49/428
Chick Embryonic	0			(0) 0/49/428
	7	(+++)	192/323/97	(0) 0/99/292
Mouse Embryonic	0			(0) 0/12/900
	10	(+)	68/194/459	(0) 0/144/618
	14	(++)	282/372/328	
	18	(+++)	271/278/81	(0) 0/156/688
HeLa‖	0			(0) 0/372/11070
	3	(+++)	577/2010/2560	(0) 0/1540/7150
FL‖	0			(0) 0/380/7290
	3	(+++)	664/2310/1880	(0) 0/220/5270
Human Embryonic	0			(0) 0/16/295
	7	(+++)	130/276/58	(0) 0/154/464

*, ‡, § See corresponding footnotes in Table I.
‖ Cell-associated forms of lipovirus were used.

cultures were fractionated at the beginning of each experiment (considered as zero day). The remainder were treated with various agents. The treated cultures were fractionated at intervals indicated in the results. Appropriate controls were included in each experiment.

RESULTS

DNA and Thymine Degradation in Infected Human "Liver" Cells.—Similar to the cell-associated form described previously (3), the cell-free form of the lipovirus induced the appearance of DNA and thymine catabolic activity in the human "liver" cells as demonstrated in Table I. The association of these catabolic activities with cytopathic changes and the retention of 2.5 to 20 per cent of total radioactivity in the acid-insoluble fraction are points of interest.

The latter finding seems to correlate with the cytological evidence of incomplete DNA degradation in the end stage of degeneration (1).

Degradation in Other Cultured Cells.—To determine if the lipovirus would induce DNA and thymine degradation in other cultured cells, degradation

TABLE III

Failure to Demonstrate DNA and Thymine Degradation in Thymidine-2-C^{14}-Labeled Cultures Killed by Freeze-Thawing

Cell*	Day	Radioactivity‡ in cell fractions	
		Freeze-thawed§	Not freeze-thawed
Liver	0		0/5/1528‖
	3	0/14/1538	0/4/1376
FL	0		0/0/2550
	3	0/0/1592	0/0/1822
PA	4	0/46/424	0/193/336
PA	4	0/114/312	0/28/276
HE	0		0/15/296
	4	0/334/241	0/150/440
HE	4	0/356/465	0/45/288
HE	7	0/220/180	0/167/162
ME	0		0/15/910
	7	0/11/618	0/144/608
CE	0		0/23/2135
	7	0/197/1764	0/114//1260

* Total number of experiments were 4, 1, 2, 4, 2, and 1 for liver, FL, primary human amnion (PA), human embryonic (HE), mouse embryonic (ME), and chick embryonic (CE) cultures, respectively; results were consistent.

‡ See corresponding footnotes in Table I.

§ Cultures killed by freeze-thawing on 0 day and then incubated at 37°C for 3 to 7 days.

‖ See corresponding footnotes in Table I.

study was repeated using human embryonic, primary human amnion, HeLa (5), FL (6), primary chick embryonic, and primary mouse embryonic tissues. Results are shown in Table II; degradations of DNA and thymine occur in all cultures killed by the lipovirus. Cultures prepared from 4 human embryos, 3 human placentae, two lots of chick, and two lots of mouse embryos have been tested with similar results. Experiments with the HeLa and FL cells

have not been repeated, however. The failure to demonstrate the degradation of thymidine-2-C^{14} to $C^{14}O_2$ in all uninfected cultures should be noted; this is contrary to the statement that in the mammals, uracil and thymine are rapidly reduced and the pyrimidine ring is then cleaved (7). Of some interest is the fairly consistent increase of the radioactivity in the acid-soluble fractions of the various uninfected embryonic tissues fractionated on the 7th as compared to the zero day.

Four infected primary amnion cultures, which were excreting viruses but showed no progressive degeneration, catabolized thymidine-2-C^{14} either not at

TABLE IV

DNA and Thymine Degradation in Thymidine-2-C^{14}-Labeled Liver Cultures Killed by Various Chemicals

Lethal agent*	Radioactivity‡ in cell fractions on day			
	0	1	4	6
None	0/398/10850	0/239/11600§	0/492/7020	0/525/7150
Actinomycin D		0/327/11600	0/454/8250	0/372/10200
Mitomycin C		0/1035/10850	0/1180/12750	0/1410/9450
FUDR			0/133/11300	0/190/10520
None	0/53/13241			0/736/14316
Actinomycin D				0/536/17474
Mitomycin C				0/1081/17050
FUDR				0/152/14911

* Used at concentration of 50 μ per ml medium; complete cell destruction occurred within 48 hours after addition of actinomycin D or mitomycin C; in culture receiving FUDR, degeneration was advanced on the 6th day.

‡, § See corresponding footnotes in Table I.

all or only at very low rates. In the presence of 0.5 μc thymidine-2-C^{14} per ml medium, only 4, 10, and 20 counts/min were detected in the CO_2 evolved during a 24 hour period; under similar conditions, 1488, 3364, 4884, and 1568 were detected in lipovirus-infected "liver" cultures showing advanced degeneration.

Degradation on Cells Killed by Various Physical and Chemical Agents.—To determine if DNA and thymine degradations are secondary to all forms of cell death, degradation experiments were performed using various lethal agents. Table III shows that no consistent DNA or thymine degradation were detectable in "liver," FL, primary amnion, and chick or mouse embryonic cultures killed by 3 cycles of freeze-thawing. With the human embryonic cultures, however, there are consistent increases in the radioactivity of the acid-soluble fractions of freeze-thawed as compared to control cultures; this finding suggests that increases in the DNA degradative activity do occur in human embryonic cultures killed by freeze-thawing.

Table IV shows the results of degradation experiments in which cells were killed by mitomycin C, actinomycin D, and 5-fluorodeoxyuridine (chemicals known to interfere with DNA metabolism). Catabolism of thymine to CO_2 cannot be demonstrated in all instances. Degradation of DNA occurs in liver cells killed by mitomycin C. This is in agreement with the reported depolymerization of DNA in mouse fibroblast strain L treated with mitomycin C (8).

TABLE V

Summary of all Degradation Study of Various Cultured Cells Infected by 13 Different Viruses

Virus	Cell system*	CPE‡
Vaccinia	Liver (2), FL (1), HA (1), HE (2), ME (2)	2 to 4
Adeno 3	" (3)	4
Herpes simplex	" (2), HE (2), ME (2)	2 to 4
Polio 1	" (2)	2
Polio 2	HE (2)	3
Coxsackie B1	Liver (2), HA (1)	2 to 4
Parainfluenza 1	CE (2)	None§
Sindbis	CE (2)	1
Rabies	CE (2), ME (2)	None§
Rous sarcoma	CE (1)	7‖
Encephalomyocarditis	ME (2)	1
Theiler's	ME (2)	None§
Polyoma	ME (2)	14¶

* HA = human amnion, HE = human embryonic, ME = mouse embryonic, CE = chick embryonic. (2) = 2 degradation experiments.

‡ Complete cytopathic changes appeared on day following infection with dosage used.

§ No definite progressive cell destruction by the 7th day, though cultures were more granular and showed more degenerated cells than control culture.

‖ Numerous "Rous foci" but no cell destruction on 7th day.

¶ Cell destruction estimated at 20 to 50 per cent on 14th day.

Degradation in Cells Infected by Other Viruses.—Degradation experiments were carried out to determine if various other viruses and rickettsia would also induce DNA and thymine degradation in cultured cells. Cell systems and viral agents employed are summarized in Table V. The total number of experiments using a particular cell-virus system together with the resulting cytopathic changes are also shown in Table V. The results are summarized in Table VI. Since no $C^{14}O_2$ was demonstrated in any of the infected cultures, the results are expressed simply as percentage of total radioactivity residing in the acid-soluble fraction. A higher percentage would indicate a greater extent of DNA degradation. Since the results vary considerably from experiment to experiment, even in the uninfected controls, an arbitrary criteria was adopted.

A culture was considered to have shown a significant increase in DNA-degrading activity when the percentage of radioactivity residing in the acid-soluble fraction was reproducibly 2 or more times that of the uninfected control of the same experiment. Based on this criteria, only mouse embryonic culture infected by the encephalomyocarditis virus shows significant increases in DNA-degrad-

TABLE VI

DNA Degradation in Thymidine-2-C^{14}-Labeled Cultures Infected with Various Viruses*

Culture‡	Day	Per Cent radioactivity in acid-soluble fractions						
		None	Vac.	Herpes	Adeno 3	Polio	Cox. B1	
Liver	3	0.3	1	1	4	1	1	
"	3	2	0.5	3	1	1	1	
"	7	5		6				
HA	4	25	6			2	16	
HE	5	25		17		26		
"	5	14	10	35				
"	6	51		72				
			Sind.	Rous	Para 1	Rabies		
CE	7	25	23	23	38	18		
"	7	11	22		16	14		
			EMC.	Theiler's	Herpes		Vac.	Polyoma§
ME	7	19	82	16	10	26	21	18
"	7	17	38	13	16	27	14	

* Vac. = vaccinia, Herpes = herpes simplex, Sind. = sindbis, Rous = Rous sarcoma, para 1 = para influenza 1, Cox. B1 = Coxsackie B1, and EMC. = encephalomyocarditis.

‡ HA = human amnion, H.E. = human embryonic, CE = chick embryonic, and ME = mouse embryonic.

§ Fractionated 14 days after infection.

ing activity. It is quite possible that some increase in DNA-degrading activity may be present in many of the other virus-cell systems. The variability of results, the limited number of experiments performed, and the limitation of the methodology preclude any conclusive statement.

Effect of DNAse on DNA Degradation.—Our failure to demonstrate more extensive DNA degradation seems contrary to the generally accepted finding of the ubiquitous presence of DNAse in living cells (9). There are at least 2 possible explanations: (a) DNA-degrading activity in cultured cells is low or absent, or (b) most of the DNA exists in complex forms insusceptible to DNAse (10, 11). An experiment was designed to test these possibilities.

A replicate set of thymidine-2-C^{14}-labeled cultures was divided into 3 groups of 2 cultures each. Group 1 cultures were killed by freeze-thawing, group 2 killed by virulent viruses, while group 3 were maintained as healthy control. To one of the 2 cultures of each group, 0.5 mg of DNAse was added. After 7 days at 36°C, all cultures were fractionated. Acid-insoluble fractions obtained in these experiments were washed 3 times in order to obtain true values for DNAse-resistant DNA.

The results shown in Table VII clearly demonstrate that at least 80 per

TABLE VII

Degradation of DNA following Addition of DNAse to Thymidine-2-C^{14}-Labeled Cultures Killed by Freeze-Thawing or Viruses

Lethal agent	DNAse	Radioactivity in cell fractions*		
		CE	ME	Liver
	mg			
None	0	0/114/1260	0/3430/9050	0/736/14316
	0.5	0/558/1044	0/3868/14904	0/3868/11714
Freeze-thawing	0	0/197/1764	0/1450/17600	0/125/19713
	0.5	0/1563/43	0/13840/266	0/11642/389
Sindbis virus	0	0/429/1548		
	0.5	0/1482/316		
Herpes virus	0		0/2485/13341	
	0.5		0/10634/641	

* Fractionated on 7th day. 0 day radioactivity was 0/23/2135, 0/84/15136, and 0/15/14911, respectively, for CE (chick embryonic), ME (mouse embryonic), and liver cultures.

cent of the DNA of killed cells can be degraded in the presence of sufficiently high concentration of DNAse. It is also interesting that 3 to 20 per cent of the DNA was not degraded even in the presence of 0.5 mg crystalline DNAse per ml medium.

DISCUSSION

Data presented indicate that the degradation of thymine-2-C^{14} with formation of $C^{14}O_2$ occurs only in cultures infected with the lipovirus. Presumably the same mechanism of reductive catabolism of pyrimidine described for certain bacteria and tissue slices is involved (12, 13). Our consistent failure to detect any conversion of thymine to CO_2 in cultured human "liver," embryonic, amnion, mouse embryonic, and chick embryonic cells, either alive, killed by physical and chemical agents, or infected by a variety of viral agents, sug-

gests the possibility that the genetic information for the synthesis of one or more enzymes necessary for these catabolic reactions is absent in these cultured cells. The consistent appearance of active thymine degradation in these same cultures infected by the lipovirus suggests to us the possibility that the missing genetic information is introduced by the lipovirus. Obviously our data are insufficient to exclude a second possibility that the genetic information for these catabolic enzymes is present in uninfected cells and the degradation mechanism is "activated" by the lipovirus through enzyme induction, removal of suppressor gene, or removal of enzyme inhibitors. Technic available at the present time, however, does not permit any conclusive proof of the validity of either possibility. The general problem associated with virus-induced acquisition of metabolic function has been reviewed recently (14).

Based on the criteria and methodology used in our experiments, increases in the DNA-degrading activity were demonstrated in human embryonic cultures killed by freeze-thawing, liver culture killed by mitomycin C, mouse embryonic culture killed by the encephalomyocarditis virus as well as in all cultures killed by the lipovirus. Since freeze-thawing, mitomycin C, encephalomyocarditis, and lipovirus represent a rather unrelated group of lethal agents, it seems reasonable to conclude that these agents "activate" the DNAse which pre-exists in the cultured cells. The activation may operate through enzyme induction and/or removal of DNAse inhibitor. This concept is in agreement with the well established finding that DNAse activity can be extracted from most living cells (9). It should be emphasized that many of the other lethal agents may have induced a definite increase of DNAse activity in the treated cells but the increases are too small to be detected by our technique.

Two other incidental findings deserve emphasis. First: none of the cultured cells used in these experiments detectably degrade thymine to CO_2; this is contrary to the concept that pyrimidines are rapidly reduced and cleaved in the mammals (7). It should be also noted that rapidly growing (livers, embryonic tissues) as well as dormant (primary amnion) cells were included in this study. Second: at least 3 per cent of cell DNA remained undegraded even after prolonged incubation with high concentration of DNAse; this suggests that some of the DNA of these cultured cells exists in complexes with RNA which are not degradable by DNAse (10, 11). To the best of our knowledge, extensive degradation of host DNA by viruses has been described only for a few bacterial viruses (15). Induction of reduction catabolism of thymine in a host cell by a virus has hitherto not been described.

SUMMARY

DNA and thymine degradation on cultured human, mouse, and chick cells were studied. Significant increase in DNA-degrading activity was demonstrated in human embryonic cells killed by freeze-thawing, liver cells killed with mitomycin C, mouse embryonic cells infected with encephalomyocarditis

virus, and in all cells killed by the lipovirus. Twelve other viral agents, actino-
mycin D, and 5-fluorodeoxyuridine failed to produce a similar increase.

Thymidine-2-C^{14}-labeled cultures, either live, killed, or infected by 19 differ-
ent physical-chemical and biological agents, did not release detectable quantity
of $C^{14}O_2$. Following infection with the lipovirus 20 to 60 per cent of the total
radioactivity of thymidine-2-C^{14}-labeled cultures was liberated as $C^{14}O_2$. It
was postulated that the lipovirus introduced into the host cells the missing
genetic information necessary for the synthesis of one or more enzymes respon-
sible for the reductive catabolism of thymine.

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