MASTER

THE RELATIONSHIP OF DNA REPAIR PROCESSES
TO
MUTAGENESIS AND CARCINOGENESIS
IN
MAMMALIAN CELLS

Progress Report
for Period November 1, 1979 - October 31, 1980

Helen H. Evans
Helen H. Evans
Case Western Reserve University
Cleveland, Ohio 44106

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ABSTRACT

The objective of this research is to determine the role of DNA repair in mutagenesis and carcinogenesis in mammalian cells. We aim to vary repair capabilities by (1) using wild-type and repair-deficient cells; (2) imposing various recovery periods following mutagenic treatment; (3) exposing cells to inhibitors of DNA repair following mutagenic treatment; and (4) varying the mutagenic treatment to cause DNA lesions repairable by different pathways. In the last year some progress has occurred along each of these approaches.

(1). We have initiated use of the host-cell reactivation viral suicide enrichment procedure in the isolation of repair-deficient mutants. Lightly mutagenized BHK cells have been infected with irradiated Herpes simplex virus (HSV); several radiation-sensitive strains have been isolated among the survivors of the infection. The characterization of these strains is in progress, and the enrichments are continuing.

(2). We have found that alterations in the frequency of mutation of C3H/10T 1/2 cells, occurring as a result of holding the cells in a confluent state following treatment with ethylmethane sulfonate, parallel the alterations in the frequency of neoplastic transformation.

(3). The repair capabilities of BHK cells have been found to be intermediate in comparison to repair-proficient and -deficient human cells with regard to the reactivation of HSV treated with various inactivating agents. The effect of confluency and of low serum levels on DNA synthesis, as well as the response to the cytotoxic effects of MNNG and acriflavin have been determined in BHK cells in preparation for the investigation of the role of DNA repair in mutagenesis and transformation.

(4). We have found that C3H/10T 1/2 cells partially recover from the toxic effects of 4-nitroquinoline-1-oxide if they are held in a confluent state for 6-22 hours following treatment. Addition of catalase did not alleviate the toxic effects of 4-NQO. The cells contain a relatively high endogenous level of this enzyme.

Time devoted by Principal Investigator in Current Term, (previously devoted and expected for remainder): 20%

Publications

Glazier, K.G., M. Weber, and H.H. Evans, Host-Cell Reactivation in Herpes Simplex Virus in BHK Cells, Radiation Research 83, 425, 1980 (Abstract).

Evans, H.H., L.M. Wilkins, M.F. Horng, C. Santoro, T.E. Evans, and K.G. Glazier, Mutagenesis and Transformation of C3H/10T 1/2 Cells Treated with Ethylmethane Sulfonate, draft of manuscript enclosed (Appendix A).

Glazier, K.G., M. Weber, and H.H. Evans, Host-Cell Reactivation of Herpes Simplex Virus in BHK Cells, draft of manuscript enclosed (Appendix B).

Objective

The objective of this research is to determine the role of DNA repair in mutagenesis and carcinogenesis in mammalian cells. Accordingly, we aim to compare repair-proficient and repair-deficient cells with regard to the frequencies of mutation and neoplastic transformation following treatment with various mutagenic/carcinogenic agents and/or environmental pollutants. Baby hamster kidney (BHK) cells will be treated with alkylating or arylaminating agents. Mutation frequency will be measured in terms of the induction of ouabain-resistant cells, while the frequency of transformation will be measured in terms of the induction of cells able to undergo anchorage-independent growth. Repair capabilities will be varied by (1) the use of wild-type and repair-deficient cell lines, the latter to be selected by a host-cell viral reactivation suicide technique; (2) the imposition of a recovery period following the mutagenic treatment; and (3) the use of inhibitors of various DNA repair processes. The correlation of specific repair deficiencies with alterations in the frequencies of mutation and/or neoplastic transformation should yield valuable information as to the role of DNA repair in mutagenesis and transformation as well as to the chemical nature of the lesions involved.

PROGRESS REPORT

Period reported: November 1, 1979 to October 31, 1980

1. Treatment of cells with 4-Nitroquinoline-1-Oxide (4-NQO) under Various Conditions

4-NQO undergoes both oxidative and reductive metabolism in mammalian cells. Both pathways generate radical species capable of producing DNA damage which in turn could result in cell killing, mutation, and/or neoplastic transformation. It is therefore of interest to determine which of these radical species is responsible for DNA damage and whether different species are responsible for cell killing as opposed to mutation or transformation.

We have measured the effect of 4-NQO on stationary cultures of C3H/10T1/2 cells and determined whether or not recovery would occur if the cells were held in a state of contact inhibition for various periods following treatment. The results, shown in Table 1, indicate that no recovery occurs when these cells are held for four hours after 4-NQO treatment, but that recovery from treatment with low levels (2.5 μ M) of the drug does occur after six hours. After treatment with slightly higher levels of the drug (5 μ M), recovery occurs during a holding period of 24 hours. These results are similar to those reported by Ikenaga and Kakunaga (Cancer Res. 37, 3672, 1977), who demonstrated the recovery of 3T3 cells during holding periods of 12-24 hours after treatment with 4-NQO.

In order to determine if H_2O_2 or hydroxyl radicals formed from H_2O_2 are involved in the damaging effects of 4-NQO, we have measured the effect of catalase added during the 4-NQO treatment on cell survival. The

Table 1. Effect of 4-Nitroquinoline-1-oxide (4NQO) and Subsequent Holding Periods on the Survival of Stationary C3H/10T1/2 Cells

4 NQO Conc (μ M)	% Survival after Holding Period			
	0 hour	4 hour	6 hour	24 hour
0	100	100	100	100
0.3	69 77 56] 67	68 69 79] 72		
2.5	51		94	99
3.0	65	51		
5.0	36 22] 29	13	31	124
7.5	8	9		
10.0	1.4	0.48		

Stationary phase cells were treated with the medium containing the above concentrations of 4 NQO for 10 minutes and were then rinsed two times with phosphate-buffered saline (PBS) and harvested immediately or incubated in normal medium for the times indicated above before harvest. Following harvest, cells were diluted, plated and incubated for two weeks, when colonies were counted. Each value in the table represents the mean of 3-10 plates.

results, shown in Table 2, indicate that the addition of catalase during 4-NQO treatment did not protect the cells from the lethal effects of the drug. In fact, survival was lower with the enzyme present than in its absence. The lack of protection could be due to either the impermeability of the cells to the added enzyme or to the presence of an excess of endogenous catalase in these cells. We have compared catalase and peroxidase activities in three cell lines, using a H_2O_2 sensing oxidase electrode (Clark, Methods in Enzymology 56, 448, 1979). The results, shown in Table 3, indicate that C3H/10T1/2 cells contain a relatively high concentration of catalase. It is not surprising, therefore, that exogenous addition of the enzyme did not offer protection. (We are indebted to Dr. J. Biaglow and Dr. M. Varnes for the catalase determinations.) Further experimentation may involve the addition of hydroxyl radical scavengers to determine whether or not these species are involved in the damaging effects of 4-NQO.

2. Effect of Alkylating Agents on Survival, Mutagenesis and Transformation.

We have previously determined that a four-hour holding period following treatment of C3H/10T1/2 cells with low levels of ethylmethane sulfonate (EMS) resulted in an increase in survival as well as an increase in the frequency of transformation, as compared to cells replated immediately after EMS treatment. In the past year, we have obtained data that indicate that the frequency of mutation to ouabain resistance also increases during the four-hour recovery period. The increase in the frequencies of mutation and transformation during short holding periods has also been reported by Terzaghi and Little (Nature 253, 548, 1975) and Chan and Little (Abstracts of the 6th International Congress of Radiation Research, Tokyo, 1979, p. 179), who treated C3H/10T1/2 cells with ionizing and ultraviolet radiation, respectively, and held the cells in a

Table 2. Effect of Exogenous Catalase on the Toxic Effects of 4 NQO in BHK Cells.

4 NQO Conc μM	% Survival	
	without catalase	with catalase
0	100	126
1.0	83	38
3.0	22	23
10.0	5	1

Log phase cells were treated with 4 NQO + catalase (10 μg/ml) for 10 min. The cells were then rinsed two times with PBS, and were harvested immediately, diluted, and plated for the determination of survival. Each number represents the average of 6 plates.

Table 3. H_2O_2 Degradation by Peroxidase and Catalase in Three Cell Lines

Cell Line	Rate H_2O_2 Degradation (nmoles/min)				Ratio Catalase/peroxidase
	Total Breakdown Catalase plus Peroxidase	Breakdown Due to Catalase	Breakdown Due to Peroxidase	No Breakdown	
	(+ glucose)	+ NEM No glucose	+ azide + glucose	+ azide + NEM No glucose	
C3H/10T1/2	3.47	1.74	1.71	0.	1.02
	1.89	0.82	1.02	0.03	0.80
Ehrlich Ascites Cells	1.93	0.01	1.86	0	0.005
	1.84	0.02	1.81	0	0.011
Hep-2	2.49	0.79	2.02	0.05	0.38
	2.70	0.88	2.43	0.07	0.36

2×10^6 cells in 3 ml PBS were preincubated +/- 1 mM glucose, 0.05 mM NEM and/or 0.2 mM azide as indicated above and 5 nmole H_2O_2 (an amount similar to that generated during the aerobic metabolism of nitroaromatic drugs). H_2O_2 disappearance was measured with a peroxide electrode. These experiments were carried out by Dr. M.E. Varnes and Dr. J.E. Biaglow.

confluent state for various periods thereafter. If the cells were held for longer periods following treatment with either radiation or with EMS, the frequencies of mutation and transformation declined from the four-hour values. The results that we have obtained following treatment with EMS are summarized in Table 4. The increased mutation and transformation frequencies seen following short holding periods in which DNA synthesis does not occur could be due to the occurrence of an error prone excision repair process (Hanawalt, Cooper, and Ganesan, *Ann. Rev. Biochem.* 48, 783, 1979). Alternatively, the increased frequencies could be due to a difference in the kinetics of the repair of potentially lethal lesions in comparison to the repair of potentially mutational and transformational lesions. Thus, if lethal lesions were repaired in cells still sustaining mutational and transformational lesions, the frequencies of the latter would increase in the surviving population. Upon further holding, these lesions in turn would be repaired, and the frequencies of mutation and transformation would decrease. These results are presented and discussed in more detail in an accompanying manuscript (Appendix A).

We have also initiated experiments to determine the effect of another alkylating agent, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) on the survival of baby hamster kidney (BHK) cells. We wish to determine whether or not holding recovery occurs in BHK cells after treatment with MNNG and if so, whether the frequencies of transformation and mutation are altered by repair occurring during this period. If the frequencies are changed during recovery, we will determine whether the removal of specific DNA alkylation products occurs during the recovery period and whether or not both the alteration of frequencies and the removal of specific alkylation products can be prevented by inhibitors of repair (such as acriflavin). We hope thus to correlate the repair of a specific lesion with the alterations in mutation and transformation frequencies.

TABLE 4

EFFECT OF HOLDING TIME ON MUTATION AND TRANSFORMATION FREQUENCIES IN C3H/10T1/2 CELLS

Holding Condition	% Survival	Transformation Frequency $\times 10^6$	Mutation Frequency $\times 10^6$
<hr/>			
A. Confluency			
0 hours	42	3.6	-
4 hours	60	6.2	-
B. Confluency			
0 hours	59	51	6.0
4 hours	63	95	13.0
24 hours	66	57	3.8
C. Log-Phase			
10% serum, 6 hours	51	4.0	3.0
0.1% serum, 6 hours	40	4.0	3.0

Cells were treated with 10 mM EMS as described in the text. Transformation frequency was measured by the fraction of cells forming colonies in soft agarose: Each group consisted of 25 dishes with 1×10^5 treated or control cells per dish. Mutation frequency was measured as the fraction of cells forming colonies in the presence of 1 mM ouabain. The frequencies have been corrected for the reduction in survival of the EMS-treated cells and for spontaneous transformation and mutation occurring in the non-EMS treated control cells.

To date we have determined the effect of various levels of MNNG and of acriflavin on the survival of stationary BHK cells. These results are shown in Figure 1 and Table 5. Experiments designed to determine whether or not BHK cells recover if held in stationary phase for 22 hours following treatment with MNNG are as yet inconclusive. BHK cells are difficult to hold in a confluent state, due to peeling of the monolayer. Figure 2 shows the increase in cell density and the incorporation of ^3H -thymidine by these cells as a function of incubation time. As can be seen in this figure, both ^3H -thymidine incorporation and the cell number decreased rapidly after confluency was reached. We have therefore tested the possibility of holding these cells in medium containing a low concentration of serum as an alternative to holding them in a confluent state. Table 6 shows that when the cells were incubated in medium containing only 0.3% serum, incorporation of ^3H -thymidine and cell division were blocked. When the low-serum medium was replaced with medium containing 10% serum, the cells were found to resume DNA replication within 24 hours. Experiments soon to be carried out will test whether or not recovery occurs when cells treated with MNNG are incubated for various periods in medium containing 0.3% serum.

3. Reactivation of Herpes Simplex Virus by BHK Cells

Another approach to accomplishing the objectives of this research is to test repair-deficient mutants for alterations in their ability to be mutagenized and transformed by chemical agents. Our objective is to isolate such mutants using a host-cell viral reactivation suicide technique. We have decided to utilize BHK cells for this project since they are diploid, thus increasing the possibility the isolation of strains which express recessive mutations over that obtained with hypertetraploid cells such as the C3H/10T1/2 cell line. Strains of BHK cells expressing recessive mutations have been isolated previously (Meiss and

Figure 1. Effect of MNNG on the Survival of BHK Cells

Stationary phase cells were exposed to MNNG for one hour at a concentration as indicated above in $\mu\text{g/ml}$. After treatment, the cells were rinsed two times with PBS and were harvested, diluted in duplicate and plated for the measurement of survival. Colonies were counted in 10 days. Each point represents the mean survival of cells plated in 10 dishes.

Table 5. Effect of Acriflavin on BHK Cells

Acriflavin Concentration μg/ml	% Survival
0	100
0.3	88
1.0	76
3.0	30

Cells were treated with medium containing the specified concentration of acriflavin for 22 hours and were then rinsed two times with PBS and plated for survival. Colonies were counted after 10 days. Each value above represents the mean of 5-10 plates.

Figure 2. Cell Division and ³H-Thymidine Incorporation by BHK Cells.

At various times after seeding cells were incubated with ³H-thymidine (0.1 µCi/ml, 10 nM) for 1 hour, and were then rinsed, harvested, enumerated, and extracted with cold and hot 0.6N HClO₄. The H and DNA content of the hot acid fraction was determined using scintillation counting and the diphenylamine reaction, respectively.

o-o: cells per cm². x-x: cpm/µg DNA.

Table 6. Effect of Serum Concentration on Cell Division and ^3H -Thymidine Incorporation in BHK Cells.

	Total Cell Number/Dish $\times 10^{-6}$	Specific Activity DNA (cpm/ μg after 1 hr pulse)
A. 0 time, 10% serum	$\begin{array}{c} 3.0 \\ 2.6 \end{array} \quad 2.8$	$\begin{array}{c} 783 \\ 651 \end{array} \quad 718$
B. 22 hours, 10% serum	$\begin{array}{c} 4.6 \\ 7.0 \end{array} \quad 5.8$	$\begin{array}{c} 447 \\ 731 \end{array} \quad 588$
C. 22 hours, 0.3% serum	$\begin{array}{c} 3.7 \\ 4.4 \end{array} \quad 4.0$	$\begin{array}{c} 71.6 \\ 182 \end{array} \quad 127$
D. 22 hours in 0.3% serum, 10 hours in 10% serum	$\begin{array}{c} 2.5 \\ 5.6 \end{array} \quad 4.0$	$\begin{array}{c} 441 \\ 426 \end{array} \quad 433$
E. 22 hours in 0.3% serum, 20 hours in 10% serum	8.1	652

Replicate flasks containing early log phase BHK cells were incubated in medium containing ^3H thymidine (1.0 $\mu\text{C}/\text{ml}$) for 1 hour at the beginning of the experiment (A), after 22 hours in normal medium with 10% serum (B) or after 22 hours in normal medium with 0.3% serum (C). In experiments D and E, cells which had been incubated in medium containing 0.3% serum were switched back to medium containing 10% serum. ^3H -thymidine was added for 1 hour after 10 hours (D) or 20 hours (E) incubation in the medium containing 10% serum.

Basilico, Nature New Biol. 239, 66, 1972; Caboche, Genetics 77, 309, 1974; Bouck and di Mayorca, Nature 264, 722, 1976). The virus to be used in the suicide enrichment procedure is Herpes simplex virus Type 1 (HSV). We have measured the reactivation of irradiated and EMS-treated HSV by BHK cells in order to determine the proper doses for use in the enrichment procedure and in order to estimate the repair capabilities of the BHK cells. Thus, we have measured the reactivation of irradiated HSV in BHK cells as compared to normal and repair-deficient human diploid fibroblasts and excision-deficient mouse cells. BHK cells were found to have an intermediate ability to reactivate UV-irradiated HSV: Reactivation was less than that observed in normal human fibroblasts, but more than that observed in xeroderma pigmentosum (XP) group A cells and mouse L929 cells. In contrast, BHK and normal human cells were equally able to reactivate HSV treated with ionizing radiation (the D_0 for the virus was 45 krad in both hosts), whereas xeroderma pigmentosum cells were slightly less efficient in the repair of Y-irradiated virus ($D_0 = 38$ krad). The effect of EMS on plaque formation by HSV on a monolayer of BHK cells is shown in Fig.3. The reactivation experiments are described in more detail in an accompanying manuscript (Appendix B).

4. Isolation of Repair-Deficient Strains: The Use of the Host-Cell Viral Reactivation Suicide Enrichment Procedure.

Small batches of BHK cells were mutagenized with EMS and grown for 10-13 generations to allow expression. Controls were treated similarly except for the addition of EMS. Samples of the mutagenized and control cells were tested for survival and for the frequency of induced ouabain-resistant mutations; the remaining cells were frozen in liquid nitrogen for future use. We have followed the procedure of Shiomi and Sato (Som. Cell Genet. 5, 193, 1979) for the enrichment procedure. Mutagenized and control cells were seeded in 150 mm petri

Figure 3. Effect of EMS on Plaque Formation by Herpes Simplex Virus (HSV)

A viral suspension containing 8×10^6 PFU per ml was incubated with EMS at the concentrations indicated above. After 15 min, sodium thiosulfate was added to inactivate the EMS. (Thiosulfate did not affect plaque formation by untreated virus.) The viral suspension was then diluted in medium containing 2% serum, and dilutions were added to flasks containing BHK cell monolayers. After an incubation of 18 hours, the medium was replaced with 0.6% agarose. After four days, the agarose was removed, the cells were stained with crystal violet, and the plaques were counted.

dishes (2000 cells/dish) and incubated until the colony size was approximately 50 cells. The virus, previously irradiated to a 14% survival level, was then added to the plates. After a 90 minute incubation, the non-adsorbed virus was removed and medium containing human gamma globulin was added to prevent liberated, repaired virus from infecting repair-deficient colonies. When the surviving colonies from the first infection had grown to a size of approximately 400 cells, irradiated HSV was added once more. Survivors of the second infection were isolated with cloning rings and grown up in normal medium. To date, we have isolated nine survivors from 36,000 cells infected with irradiated virus. Three of these were lost during subculture. When sufficient numbers of the remaining six strains were obtained, samples were frozen, and aliquots were tested for their sensitivity to ionizing radiation. Details of the enrichment procedures are shown in Table 7, whereas the response of the survivors to X-irradiation is shown in Fig. 4. It appears that at least two of these strains are somewhat sensitive to X-irradiation. The response of V_1 and V_2 relative to the parental cells was found to be reproducible in a second experiment, and additional tests of the other strains are in progress. Further plans for the characterization of these strains and for the continuation of the enrichment and isolation procedures are outlined in the accompanying research proposal.

TABLE 7

SUMMARY OF TWO HOST CELL REACTIVATION - VIRAL SUICIDE ENRICHMENTS

	EMS CONC. mM	% SUR- VIVAL	OUA ^R /OUA ^S (X 10 ⁶)	CELLS TREATED ^a	HSV ^b added/plate (PFU X 10 ⁻⁸)		SURVIVING COLONIES
					1st infec- tion	2nd infec- tion	
EXP. 1	0	100	1.7	4000	1	0.3	1 ^c
	2.5	119	73	4000	1	0.3	2 ^c
	10	29.4	347	4000	1	0.3	0
	20	2.0	114	4000	1	0.3	1
EXP. 2	0	100	0.83	-	-	-	-
	2.5	228	5.84	8000	1	0.3	0
	2.5			6000	0.5	0.3	2
	2.5			6000	0.3	0.3	3

^aNumber of BHK cells treated with irradiated virus. Each plate was seeded with 2000 cells.

^bThe PFU of the HSV has not been corrected for inactivation by γ irradiation.

^cDid not survive upon subculture.

Figure 4. Effect of X-Radiation on the Growth of Strains Isolated Using the Viral Enrichment Procedure.

Cells were suspended in complete medium, counted and treated with various amounts of X-radiation. Appropriate dilutions of each sample were then plated in triplicate. After incubation for ten days, colonies were counted. The survival curves for the various isolates are labeled (V₁, etc.). Control cultures are included in each set (x—x).

In a second experiment, the response of V₁ and V₆ to X-radiation was similar relative to the parental cells as that shown above. Retests on the sensitivities of V₂, V₃, V₄ and V₅ are in progress.