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Rat Hepatocyte Primary Cell Culture-Mediated Mutagenesis of Adult Rat Liver Epithelial Cells by Procarcinogens[1] (39974)

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In view of the broad capability of the liver for metabolic activation of carcinogens (1), liver cells represent one of the potentially most useful culture systems for carcinogenesis studies (2). It is now possible to initiate high-viability rat hepatocyte primary cell (HPC) cultures (3) composed almost exclusively of hepatocytes (4). In addition, such rat hepatocyte primary cultures retain a high degree of the functional activity of the liver within the first 24 hr after initiation (4-8). Use of HPC cultures in carcinogen-induced DNA repair studies revealed that they can activate to DNA-damaging metabolites a wide spectrum of procarcinogens requiring different pathways of metabolism (9, 10), including polycyclic aromatic hydrocarbons, aromatic amines, azodyes, nitrosamines, and mycotoxins. Since many carcinogens are also mutagens, the use of HPC cultures in carcinogen-induced mutagenesis studies was examined. In the first 48 hr in culture HPC are nonproliferating and lack the capacity to form colonies following subculture by trypsinization. Therefore, although mutagenesis could not be studied in them by available techniques, they were used as a feeder system for providing metabolic activation (11), as has been done in other systems (12-14). The target cells in these studies were established lines of adult rat liver (ARL) epithelial cells (15) which can be mutagenized to 8-azaguanine (AG) resistance (16) and which associate closely with the HPC cells for contact feeding of activated metabolites.

In the present report, the ability of HPC cultures to serve as a feeder system providing activation of procarcinogens from three different classes is described.

Materials and methods. The preparation of high-viability dissociates of hepatocytes has been described previously (3, 4). For the continuous cultures of adult rat liver epithelial cells, ARL 6 was derived from a Wistar strain rat, while ARL 11 and 16 were derived from Fischer strain rats (15). All cell cultures were maintained in Williams Medium E (WE) (15) (Flow Laboratories, Rockville, Md.) supplemented with 10% fetal bovine serum (FBS) (Flow).

The carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) was purchased from Eastman Kodak. 2-Acetylaminofluorene (2-AAF) was kindly provided by Dr. E. Weisburger, National Cancer Institute. Dimethylnitrosamine (DMN) was obtained through the Carcinogenesis Standard Reference Compound Bank, National Cancer Institute, Bethesda, Md.

Stock solutions of the carcinogens DMBA and 2-AAF were prepared in dimethylsulfoxide (DMSO) and diluted with WE containing 10% FBS to give the appropriate final concentrations. The highest final concentration of DMSO was 0.1%. DMN was dissolved directly in WE containing 10% FBS.

For carcinogen treatment, 5.4×10^6 freshly dissociated hepatocytes and 2.4×10^6 ARL cells were premixed and then inoculated into a 75-cm^2 culture flask (Falcon Plastics, Oxford, Calif.) containing WE with 15% FBS. Control flasks contained either 5.4×10^6 hepatocytes or 2.4×10^6 ARL cells. After 3 hr, the cell cultures were washed once with serum-free WE to remove unattached cells and treated with carcinogen for 24 hr. The cells were then washed twice with serum-free WE, and maintained in medium containing 10% FBS for 24 hr for recovery and mutant expression. After this, cultures were trypsinized

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and inoculated into 25-cm^2 culture flasks (Falcon Plastics) in triplicate for determination of colony forming efficiency (CFE) and incidence of AG-resistant mutants. The percentage of colony forming cells was measured by inoculating flasks at 20 cells/cm^2 (16), refeeding once at 24 hr. When the colonies had attained a macroscopically visible size (between 9 and 12 days), they were fixed in 10% formalin and stained with Giemsa. To measure the incidence of resistant mutants, flasks were inoculated at a density of 10^4 cells/cm^2 (16). Treatment with AG (60 μg/ml) (Sigma Chemical Co.) was started 1 day after inoculation, thus allowing a total of 48 hr for mutant expression, and refed every 2 days. At 14 days, the cultures were fixed in 10% formalin and stained with Giemsa for visible AG-resistant colonies. All colonies with 32 or more cells were scored.

Results. Since the ARL cells were replicating, whereas the HPC were not, they could be identified as [^{3}H]thymidine-labeled cells in autoradiographs of mixed cultures. An autoradiograph of a mixed culture exposed to [^{3}H]thymidine (1.5 μCi/ml) for 24 hr revealed that the ARL cells were well dispersed and in close contact with the primary hepatocytes (Fig. 1). In addition, the attachment efficiency of viable cells in the mix was 48% for HPC and 44% for ARL compared to 52 and 43%, respectively, for these cells inoculated separately. Also, the percentage viability in the mix was 77% for HPC and 85% for ARL.

The HPC culture had zero capacity to form colonies following subculture at 24 hr (Table I). Control ARL cultures without carcinogen treatment possessed a spontaneous incidence of 168 AG-resistant cells per 10^6 colony forming cells. For the control HPC + ARL mixture, the number of spontaneous AG-resistant mutants was essentially the same as that observed with ARL cells alone. Treatment of the cultures with WE containing 0.1% DMSO did not increase the number of AG-resistant mutants.

Following treatment of an ARL 6 culture with the procarcinogen DMBA, there was essentially no increase in AG resistant mutants (Table I). However, when a mixed culture of HPC and ARL cells was treated with DMBA, there was a 2.3-fold increase of the incidence of AG resistant mutants over that in the DMBA-treated ARL cells.

In the same experiment, similar results were obtained with the procarcinogens DMN and 2-AAF, showing 2.4-fold and 1.5-fold increases in mutagenesis, respectively (Table I).

When the experiments were repeated with two other ARL lines as target cells, treatment of a mixture of ARL 11 or 16 and an HPC culture with carcinogen again resulted in an increase in AG-resistant mutants over that observed with the carcinogen-treated ARL cells alone (Table I, legend).

Comparison of trypsinized suspensions of HPC + ARL and ARL cultures showed that the primary hepatocytes could be readily distinguished from the ARL cells; the HPC were considerably larger in size and different in morphology from the ARL cells. Thus, in a total of 500 viable cells from a mixed ARL + HPC culture inoculated for CFE, 463 were determined to be ARL cells. Therefore, the denominator would not be significantly different for calculating the incidence of AG^r colonies whether the total cell number rather than the actual number of ARL cells in the HPC + ARL mixture was used in the computation. Considering that HPC and ARL cells were inoculated at an initial ratio of 2.25 to 1 (i.e., $5.4 \times 10^6/2.4 \times 10^6$), an increase in the relative proportion of the ARL cells to represent 90% of the retrieved cells in the suspension used for plating for CFE and AG resistance was responsible for this situation.

Discussion. The present study revealed that treatment of a mixture of ARL and HPC cultures with procarcinogens resulted in a level of mutagenesis that was not observed with carcinogen treatment of the ARL cells alone. These preliminary results indicate that the HPC culture was capable of generating mutagenic species from procarcinogens and transferring them to ARL cells. The close proximity of the HPC and ARL cells in a mixed culture would facilitate such transfer.

The use of the three procarcinogens, DMBA, DMN and 2-AAF, in this study

has provided the first demonstration of cell-mediated mutagenesis with chemical carcinogens requiring different pathways of metabolic activation (1). Previous reports on cell-mediated mutagenesis of mammalian cells with chemical carcinogens were limited to polycyclic aromatic hydrocarbons (12–14). In those studies, Chinese hamster V79 cells, which do not metabolize polycyclic hydrocarbons, were cocultivated with lethally irradiated rodent cells that can metabolize these carcinogens.

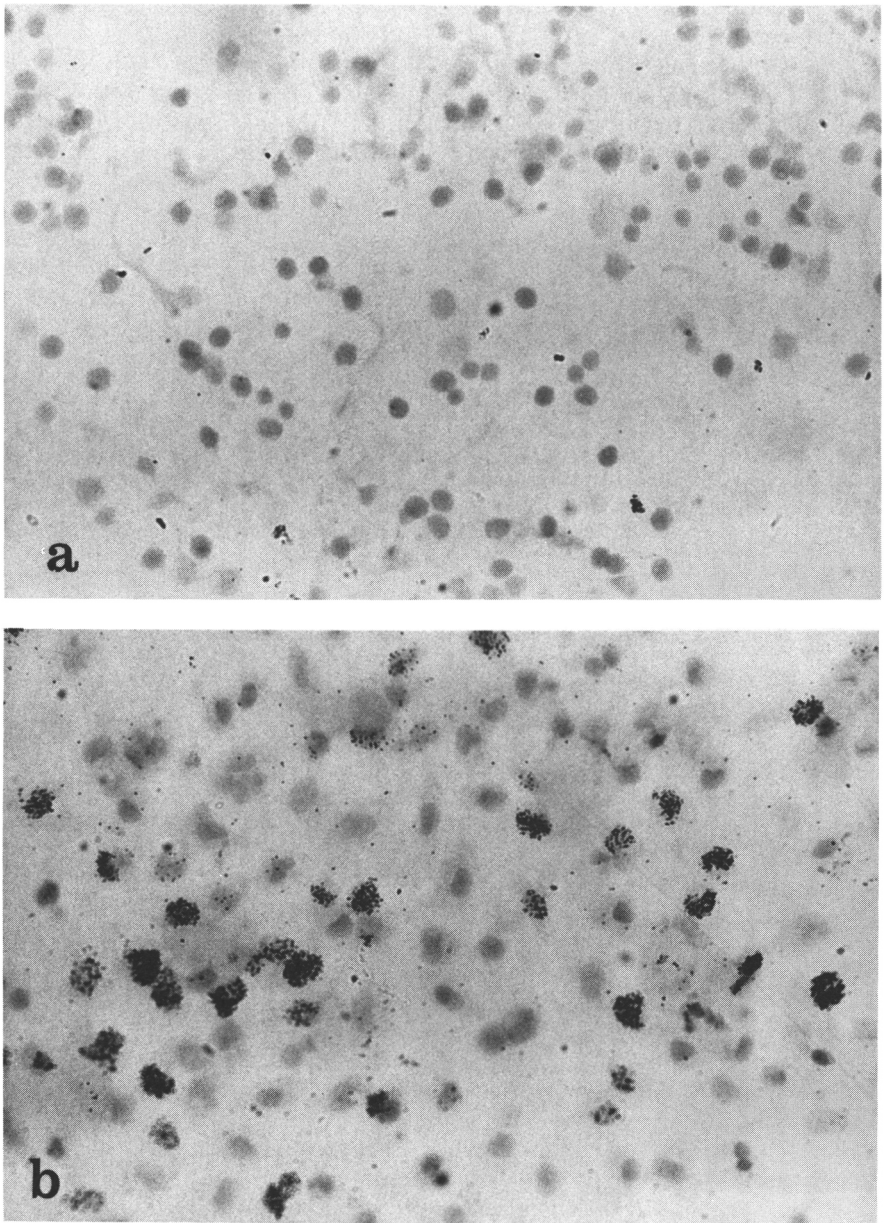

FIG. 1. Autoradiographs of rat hepatocyte primary cell culture (HPC) and adult rat liver (ARL) epithelial cells following 24-hr exposure to [^{3}H]thymidine (1.5 μCi/ml) administered at 3 hr postinitiation of cultures. (a) Nonreplicating HPC; (b) mixed HPC + ARL 6 culture, showing replicating ARL 6 cells (heavily labeled) well dispersed among the HPC cells.

TABLE I. RAT HEPATOCYTE PRIMARY CELL CULTURE (HPC)-MEDIATED MUTAGENESIS OF ADULT RAT LIVER (ARL) EPITHELIAL CELLS BY PROCARCINOGENS.

Cell culture	Treatment	Colony forming efficiency (%)	No. of AG^r cells per 10^6 colony forming cells	Increase in mutant incidence[a] (HPC + ARL)/ ARL
HPC + ARL 6	DMBA (10^{-7} *M*)	15.8	430	2.3×
ARL 6	DMBA (10^{-7} *M*)	21.4	187	
HPC	DMBA (10^{-7} *M*)	0	0	
HPC + ARL 6	DMN (10^{-3} *M*)	19.0	337	2.4×
ARL 6	DMN (10^{-3} *M*)	43.6	138	
HPC	DMN (10^{-3} *M*)	0	0	
HPC + ARL 6	2-AAF (10^{-5} *M*)	36.0	322	1.5×
ARL 6	2-AAF (10^{-5} *M*)	30.0	213	
HPC	2-AAF (10^{-5} *M*)	0	0	
HPC + ARL 6	DMSO (0.1%)	26.8	202	
ARL 6	DMSO (0.1%)	25.8	188	
HPC	DMSO (0.1%)	0	0	
HPC + ARL 6	None	27.0	207	
ARL 6	None	26.2	168	
HPC	None	0	0	

[a] The experiments were repeated three times, yielding similar increases in mutant incidence: DMBA, 2.5×, 4.1×; DMN, 2.0×, 3.1×; 2-AAF, 1.5×, 1.7×. The same experiments with two other ARL lines as target cells also resulted in an increase in AG^r mutants in ARL 16 and ARL 11, respectively: DMBA, 1.5×, 2.9×, DMN, 1.9×, 2.1×; 2-AAF, 3.2×, 3.0×.

The HPC-mediated mutagenesis system also has the advantage that the feeder cells are not subculturable and, therefore, do not have to be irradiated. However, the use of irradiated rodent cells has yielded 25- to 200-fold enhancements of mutagenesis, depending on the carcinogenic polycyclic hydrocarbon used (12–14). But, the high level of enhancement of mutant induction in those studies was observed when the CFE of the target V79 cells was very low (12–14). At levels of toxicity to the target cells comparable to those obtained in the present study (i.e., up to a 50% reduction in CFE), the enhancement of mutagenesis was about 2.8- to 16-fold (12, 13). In the HPC + ARL system, a significant though lower enhancement (1.5- to 4.1-fold) of mutagenesis was observed. The basis for the lower level of enhancement of mutagenesis is not knowr and further work on optimizing the experimental conditions (increasing mutant yield) is warranted. Nevertheless, the present results indicate that HPC cultures are useful as a feeder system for cell-mediated mutagenesis, and might be developed in this regard as a screen for environmental procarcinogens in addition to their utility for detecting DNA-damaging agents (9, 10).

Summary. Rat hepatocyte primary cell (HPC) cultures which were unable to be subcultured were used as a metabolizing feeder system for established lines of adult rat liver (ARL) cultures in studies of procarcinogen-induced mutagenesis. Treatment of a mixed culture of HPC and ARL cells with 7,12-dimethylbenz(a)anthracene, dimethylnitrosamine, or 2-acetylaminofluorene for 24 hr resulted in an increase in the incidence of 8-azaguanine-resistant mutants that was not observed with treatment of ARL cells alone. Thus, HPC cultures can be used as a feeder system for enhancing mutagenesis in established lines of ARL cells by procarcinogens requiring metabolic activation.

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