

Genotoxic Effects of 2-Acetylaminofluorene on Rat and Human Hepatocytes

by Stephen C. Strom,* Randy L. Jirtle* and George Michalopoulos*

Isolated rat and human hepatocytes in primary culture were shown to metabolize AAF to reactive intermediates which damaged hepatocyte DNA. A significant increase in unscheduled DNA synthesis was detectable by autoradiography in rat and human hepatocytes exposed to concentrations of AAF as low as 1 μM . When rat hepatocytes were plated over confluent monolayers of human fibroblasts and exposed to 3H -AAF, significant binding of AAF to the DNA of the fibroblasts as well as the hepatocytes was measured. In other experiments with hepatocyte-fibroblast cocultures, nonradioactive AAF, at concentrations greater than 40 μM , induced a significant increase in the HPRT⁻ mutation frequency in the human fibroblasts. These results demonstrate that hepatocytes can be used to assess genotoxicity of carcinogenic compounds and are useful for interspecies comparisons in chemical carcinogenesis.

Introduction

2-Acetylaminofluorene (AAF) is a potent rat liver carcinogen and is an example of a compound that is carcinogenic and mutagenic only after activation by cellular metabolic pathways to a reactive electrophile (1). The propensity of the aromatic amides and amines to induce tumors at sites distant from, but not at, the site of administration led to speculation that these compounds required metabolic conversion to other forms which were the ultimate carcinogens (1). Observations that the *N*-hydroxy metabolite of AAF was a more potent carcinogen than the parent compound implicated *N*-hydroxy-AAF as an intermediate or proximate carcinogenic form of AAF (2). The further activation of *N*-hydroxy-AAF to the ultimately carcinogenic form(s) is/are via cytosolic sulfotransferase (3), *N*-*O*-glucuronidation (4) or acetylation (5), or via an *N*,*O*-acetyl transferase reaction (6, 7).

There are marked species, sex and tissue differences in the activation and the susceptibility to AAF carcinogenesis (1, 8, 9). Guinea pigs appear to be resistant to AAF carcinogenesis due to a lack of *N*-hydroxylation activity (8) but in other resistant animals, such as the rhesus monkey, Cotton rat and X/Gf mice (8-11), the reasons for the resistance are less clear. Because of the wide variations in the activation and the sensitivities to AAF carcinogenesis

observed in experimental animals, estimates of the toxicity that would result from human exposures to AAF or other aromatic amines are difficult based on animal data. Therefore, human susceptibility to aromatic amides or amines may be investigated with human tissues.

We have developed a coculture system of rat hepatocytes and human fibroblasts (12) which is useful for investigations of carcinogen activation, DNA binding of carcinogens, DNA repair, and the mutagenesis induced in human fibroblasts by procarcinogens activated by intact rat hepatocytes (13, 14). We have recently described the conditions for the isolation and culture of human hepatocytes and have demonstrated that human hepatocytes obtained by collagenase perfusion are useful for *in vitro* studies with chemical carcinogens (15). In this report we present data which indicate that when radiolabeled-AAF is added to rat hepatocytes in culture with human fibroblasts, AAF residues are found on the DNA isolated from the fibroblasts as well as the DNA of the hepatocytes. In other experiments, when nonradioactive AAF was added to hepatocyte fibroblast cocultures, sufficient amounts of "activated" AAF were transferred from the hepatocytes to the fibroblasts to induce a significant increase in the hypoxanthine-guanine-phosphoribosyltransferase (HPRT⁻) mutation frequency in the human fibroblasts. We also present further evidence of the activation of AAF by human hepatocytes by the demonstration of significant increases in un-

*Departments of Radiology and Pathology, Duke University Medical Center, Durham, NC 27710.

scheduled DNA synthesis in human hepatocytes following exposure *in vitro* to AAF.

Materials and Methods

Cell Culture

Human male diploid fibroblasts (GM 3468 Human Genetic Cell Repository) were maintained at passages 7-12 in Eagles minimal essential medium (MEM) supplemented with amino acids and hypoxanthine/ $10^{-6}M$ amethopterin/ $10^{-6}M$ thymidine (HAT) as previously reported (13). Mutagenesis experiments and the determination of the relative plating efficiency was performed as described (13).

Hepatocytes were isolated by perfusion of the livers of male Sprague-Dawley rats (Charles Rivers, Wilmington DE) with collagenase (13). Approximately 3.5×10^6 hepatocytes were plated over the fibroblast monolayers in MEM supplemented as above and with 5% fetal bovine serum (FBS) (Sterile Systems Inc. Logan, UT) to aid in the attachment of the hepatocytes to the fibroblasts. Cultures were left undisturbed for 4 hr and then the medium was changed to serum-free MEM. AAF (Aldrich Chem Co.) was dissolved in dimethyl sulfoxide (DMSO) and was added to the cultures after the media change (1% final concentration of DMSO in the media in all experiments). After the addition of AAF, the cocultures or cultures of fibroblasts alone were incubated at 37°C for 45 hr. After the carcinogen exposure, hepatocyte-fibroblast cocultures or cultures of fibroblasts alone were trypsinized and the relative plating efficiency and the induced HPRT⁻ mutation frequency was determined as described (13).

Determination of the Binding of AAF to DNA

Binding of ³H-AAF to hepatocyte and fibroblast DNA was determined as described previously (14). Approximately 7 days prior to being overlaid with hepatocytes, fibroblast cultures were trypsinized and incubated with $10 \mu M$ 5-bromo-2-deoxyuridine (BRdU) as reported (14). BRdU was added to the media to substitute for thymidine in the fibroblast DNA. BRdU-substituted fibroblasts cultures were grown to confluency in total darkness. When the fibroblast cultures were confluent, hepatocytes were added under reduced gold light (Westinghouse F72T12 high output gold) as indicated above. After the media change, ³H-AAF (Ring ³H, 1 mCi/mg, donated by Dr. F. A. Beland, National Center for Toxicological Research) was added to the cultures in BRdU and serum-free media, either directly or with an appropriate amount of unlabeled AAF. Hepatocyte-fibroblast cocultures or cultures of fibroblasts

alone were incubated with the ³H-AAF for 45 hr in the dark. The cultures were harvested by scraping and the hepatocyte DNA was separated from the heavier fibroblast DNA as previously described (14). An additional cesium chloride isolation step with the TV-865 vertical rotor (Dupont-Sorvall) was added to insure complete separation of the normal density, hepatocyte DNA from the hybrid density, BRdU-substituted fibroblast DNA. The specific activities of the DNAs were determined as reported (14).

Unscheduled DNA Synthesis (UDS) Experiments

Isolated rat or human hepatocytes were plated on collagen-coated (12) chamber slides (Lab-Tec) for 4 hr in MEM supplemented as described above and with 5% FBS. After 4 hr the medium was changed and 1, 10 or 100 μM AAF and 7.5 $\mu Ci/mL$ of ³H-thymidine (ICN, 40-60 Ci/mmol) were added to the cultures in serum-free media. Cultures were incubated for 24 hr at 37°C in a fully humidified 5% CO₂ atmosphere. After 24 hr, the slide cultures were washed in five sequential 4L beakers of saline (0.9%), washed in 100% methanol for approximately 20 sec and fixed for 24 hr in phosphate-buffered 10% formalin (pH 7.2). Slides were dipped in Kodak NTB-3 emulsion diluted 1:1 with distilled water and were stored in the freezer in the dark for 7-10 days. The slides were subsequently developed and stained with hematoxylin and eosin. Grains over the nuclei of the hepatocytes were counted at 400 × or 1000 × (oil immersion) with a Leitz microscope.

Results

AAF activated by freshly isolated rat hepatocytes induced a significant increase in the HPRT⁻ mutation frequency in the human fibroblasts when cocultures of the two cell types were exposed to concentrations of AAF greater than 40 μM (Fig. 1B). The increase in the mutation frequency was dependent on the activation of AAF by the hepatocytes, as no significant stimulation in the HPRT⁻ mutation frequency over the control value of $0.38 \pm 0.09 \times 10^{-5}$ (mean \pm S.E.) was observed when cultures of human fibroblasts were exposed to AAF in the absence of hepatocytes ($0.37 \pm 0.1 \times 10^{-5}$ for 40-600 μM AAF). The relative plating efficiencies of the fibroblasts after exposures to the indicated concentrations of AAF are given in Figure 1A. There was a dose-dependent decrease in the plating efficiency of the fibroblasts exposed to AAF in the presence of hepatocytes (open circles). When cultures of fibroblasts were exposed to AAF in the absence of hepatocytes, there was not a significant decrease in

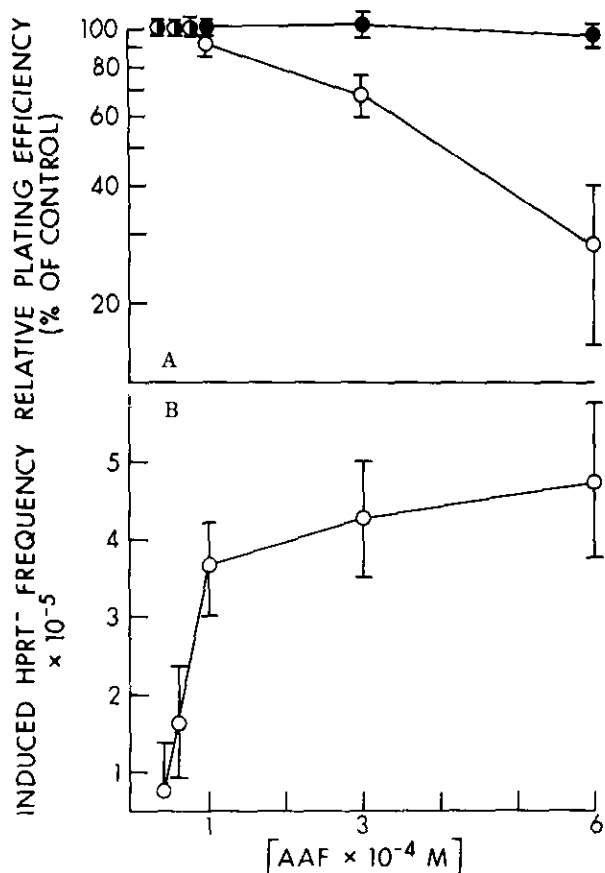


FIGURE 1. Cytotoxicity and mutagenicity of AAF. Human fibroblasts were exposed to the indicated concentrations of AAF (○) in the presence or (●) the absence of hepatocytes. (A) The plating efficiency of fibroblasts exposed to the indicated concentrations of AAF. The ordinate is in log scale and the results are expressed as a percentage of the control plating efficiency. (B) The frequency of HPRT⁻ mutant human fibroblasts is expressed as numbers of mutants per 10^5 clonogenic survivors. The control background mutation frequency was $0.38 \pm 0.09 \times 10^{-5}$ in four experiments. Data points are the means \pm the standard error of the mean from four experiments. The induced mutation frequency for fibroblasts exposed to concentrations of AAF $> 40 \mu M$, in the presence of hepatocytes was significantly different from control ($p < 0.05$) as determined by Student's *t*-test. For fibroblasts exposed to AAF in the absence of hepatocytes the induced mutation frequency did not exceed $0.37 \pm 0.1 \times 10^{-5}$.

the plating efficiency of the fibroblasts (closed circles), which is consistent with our observations that no mutations were induced in the fibroblasts by AAF unless AAF was first activated by the hepatocytes.

We investigated the binding of ³H-AAF to hepatocyte and fibroblast DNA when 10-300 μM AAF was added to the combined cultures of hepatocytes and fibroblasts or cultures of fibroblasts alone. Substantial amounts of ³H-AAF were recovered bound

to fibroblast DNA as well as to the hepatocyte DNA (Table 1). Between 5 and 15% of the AAF bound to the fibroblast DNA was due to fibroblast activation as determined by the binding of AAF to the DNA of cultures of fibroblasts alone. Binding of AAF to the DNA of fibroblasts exposed to AAF in the absence of hepatocytes was subtracted from the values for the fibroblasts presented in Table 1. Although there was a slight (but nonsignificant) increase in the binding of AAF to the fibroblast DNA as the concentration of AAF was increased in the media, there does not appear to be a clear association between the level of AAF binding to fibroblast DNA and the induced mutation frequency in these experiments. The levels of specific DNA adducts

Table 1. Binding of ³H-AAF to hepatocyte and fibroblast DNA.^a

	Binding of AAF, pmole/ μg DNA		
	[AAF] = 10 μM	[AAF] = 100 μM	[AAF] = 300 μM
Hepatocytes	12.8 \pm 4.0	18.0 \pm 2.0	34.5 \pm 8.0
Fibroblasts	138 \pm 25	141 \pm 20	165 \pm 45

^aRat hepatocytes were plated over confluent monolayers of human fibroblasts whose DNA was previously substituted with BRdU and the cocultures were exposed to 100 μM ³H-AAF for 45 hr under conditions identical to those of the mutagenesis experiments. After the exposure, normal density hepatocyte DNA was separated from the heavier BRdU substituted fibroblast DNA by equilibrium density centrifugation in cesium chloride, and the specific activity of the fibroblast and hepatocyte DNA was determined. The results shown are the means \pm SE of two or three experiments.

may correlate better with the induced mutation frequency than the overall level of binding of AAF to DNA.

Human hepatocytes were isolated and cultured as previously described (15). A light micrograph of human hepatocytes in culture for 24 hr is presented in Figure 2. The hepatocytes have attached to the collagen substrate and have spread out to form a monolayer by 24 hr. The epithelial morphology of the human hepatocytes in culture with their granular cytoplasm and prominent nuclei is very similar in appearance to rat hepatocytes. Human hepatocytes were exposed *in vitro* to 1, 10 or 100 μM AAF and unscheduled DNA synthesis was measured during a 24-hr exposure to the carcinogen and ³H-thymidine. There was a dose response increase in the amount of DNA repair measured by autoradiography in the human and rat hepatocytes exposed to AAF (Table 2). The amount of DNA repair replication measured in Case 4 was less than half that measured in Case 3 after exposures to the same concentrations of AAF. Case 4 showed less repair than that measured in Sprague-Dawley rat hepatocytes exposed to AAF under conditions identical to those used for the human cells, but the repair re-

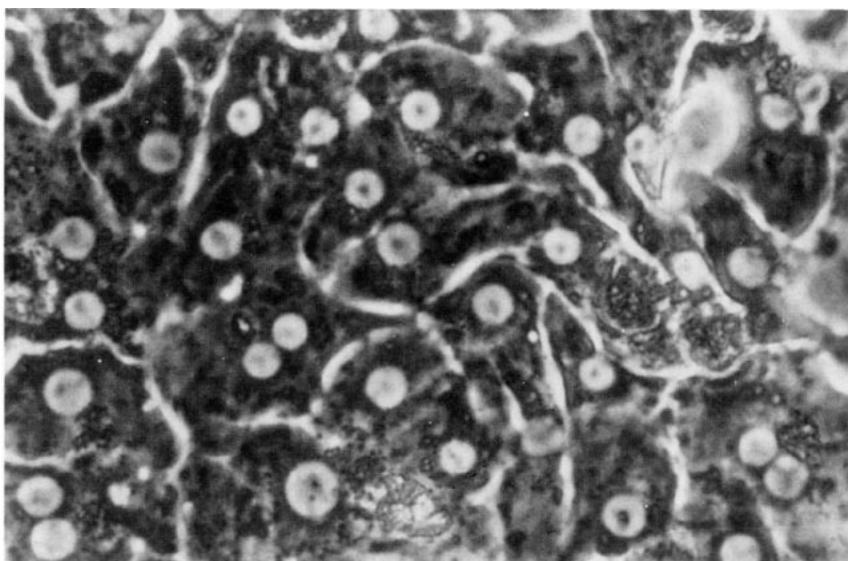


FIGURE 2. Human hepatocytes in culture. Phase-contrast light micrograph of human hepatocytes in culture for 24 hr. The hepatocytes have a fine granular cytoplasm and a prominent nuclei.

Table 2. UDS measured in rat and human hepatocytes.^a

[2-AAF], μM	Human hepatocytes		Rat hepatocytes (Sprague-Dawley)
	Case 3	Case 4	
0 (control)	3.7 ± 0.9	3.12 ± 0.34	6.4 ± 0.5
1	15.2 ± 3.2	4.5 ± 0.5	17.6 ± 1.8
10	26.5 ± 2.5	9.5 ± 0.9	29.4 ± 3.7
100	37.4 ± 2.9	14.8 ± 1.0	30.0 ± 3.1

^aFreshly isolated rat or human hepatocytes were exposed to the indicated concentrations of AAF plus ³H-thymidine, 7.5 $\mu\text{Ci}/\text{ml}$ for 24 hr. UDS measured in all carcinogen exposed cultures was significantly different from control, $p < 0.01$.

sponse observed in Case 3 was very similar to the repair response seen in the rat. Photomicrographs of the UDS measured in human hepatocytes exposed to 0 (DMSO control) or 100 μM AAF are presented in Figure 3.

Discussion

Results presented here indicate that the potent rat liver carcinogen, AAF, is activated by freshly isolated rat hepatocytes in culture and that the active metabolite(s) pass out of the hepatocytes to bind to the DNA of human fibroblasts in coculture with the hepatocytes. The amount of activated AAF that reaches the fibroblasts is sufficient to induce a significant increase in the HPRT⁻ mutation frequency in the human fibroblasts. We have also demonstrated that the binding of AAF to fibroblast DNA and the increase in the mutation frequency is dependent on the presence of hepatocytes in culture with the fibroblasts during the exposure to AAF.

These results are the first demonstration of hepatocyte-mediated AAF mutagenesis in human fibroblasts. The hepatocyte-fibroblast coculture should be of significant value in the determination of the mutagenicities of the various DNA adducts that result from the covalent interaction of AAF with DNA (16-18).

Human hepatocytes exposed to AAF *in vitro* responded to the carcinogen exposure with significant increases in UDS as measured by autoradiography. These results are in agreement with our previous observations (14, 19) and those of others (20, 21), of a stimulation of UDS in rat hepatocytes by AAF. The stimulation of UDS in Cases 3 and 4 is consistent with our previous observations of a significant stimulation of UDS in human hepatocytes by AAF (15). The amount of UDS measured in hepatocytes from Case 4 was much lower than that measured in the hepatocytes from Case 3 at all concentrations of AAF tested and lower than our previous observations of AAF-induced UDS in human hepatocytes (15). The lower relative amounts of UDS measured in hepatocytes from Case 4 relative to the other cases may be due to poorer activation of AAF by the hepatocytes from Case 4, or poorer repair of the damaged DNA by the hepatocytes in Case 4 relative to the other cases. Several fold variations in the activation of aromatic amines by subcellular fractions of human liver from different subjects has been reported by Dybing et al. (22). Hepatocytes from Cases 3 and 4 responded with UDS in a nearly identical manner after exposures to diethyl- and dimethylnitrosamine (unpublished observations). UDS induced by benzo(a)pyrene in the hepatocytes from

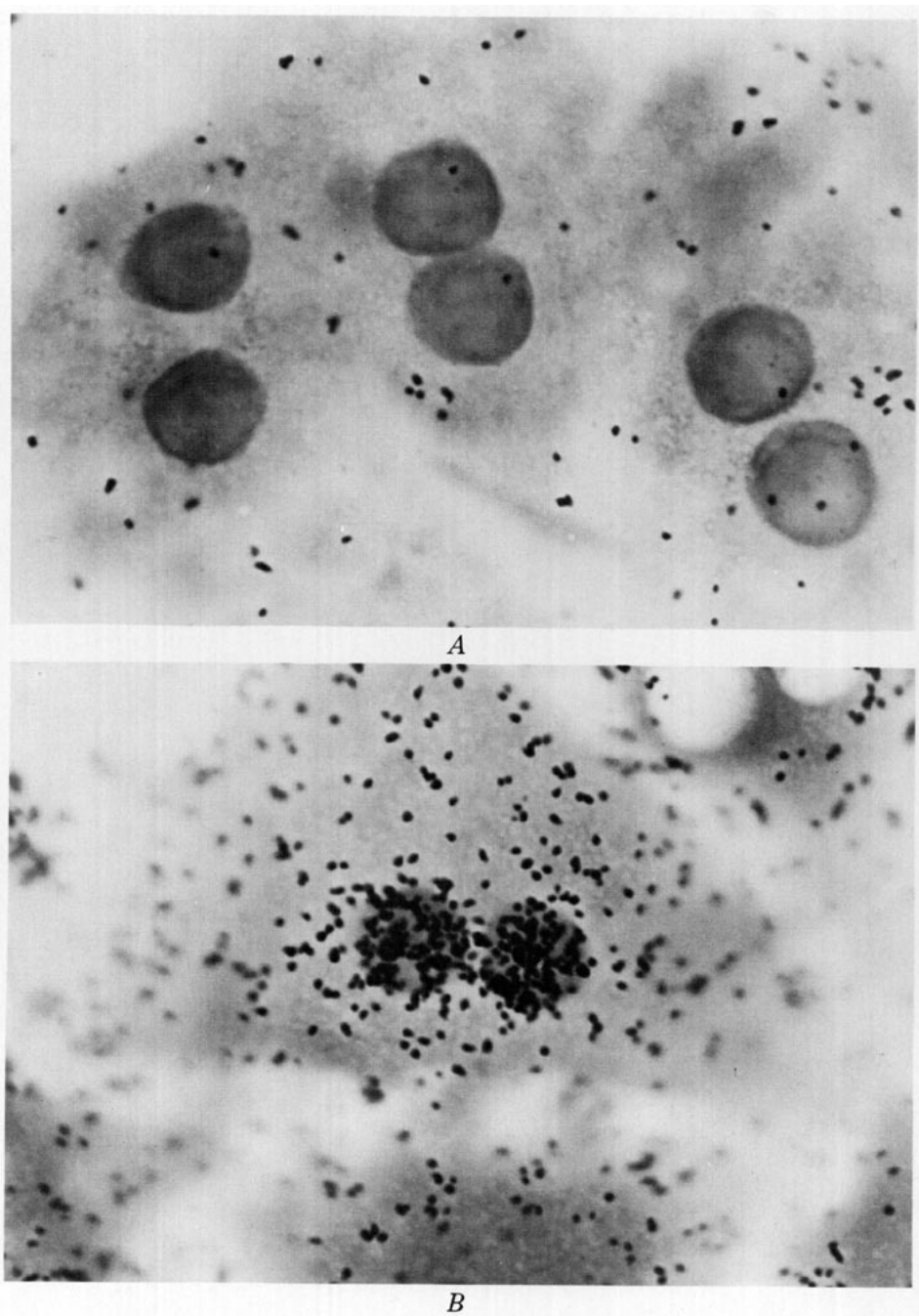


FIGURE 3. UDS measured in human hepatocytes. Hepatocytes were exposed to (A) $0 \mu\text{M}$ (1% DMSO), or (B) $100 \mu\text{M}$ AAF, for 24 hr in the presence of $7.5 \mu\text{Ci/mL}$ of ^3H -thymidine. Photomicrographs were taken at $750\times$.

Case 3 was approximately half of the value we have normally observed for human hepatocytes (15). Both AAF and benzo(a)pyrene are repaired by the long patch repair pathway (23), and it is possible that hepatocytes from this patient were slightly defective in long patch repair compared to the other cases.

Hepatocytes obtained by the collagenase perfu-

sion of pieces of human liver may be useful for the detection of chemicals carcinogenic to humans. Human hepatocytes may be used directly for the detection of potentially carcinogenic chemicals with the UDS assay as presented here and (15) or may be combined with human fibroblasts to establish an all human tissue mutagenesis system. Aust et al. (24)

have recently described the establishment of human kidney carcinoma cell-mediated human fibroblast mutagenesis system. The carcinoma cells were demonstrated to be useful for the activation of aromatic hydrocarbons to mutagenic metabolites for the human fibroblasts. Because of the extensive carcinogen activating capabilities of hepatocytes compared to other tissues, a human hepatocyte-human fibroblast coculture system may be useful for the detection of a greater number of classes of mutagenic chemicals than other cell-mediated systems.

This work was supported by National Cancer Institute grants CA06743, CA25951 and CA26904 and by grant CA30241 from the National Institutes of Health.

REFERENCES

1. Miller, J. A. Carcinogenesis by chemicals: an overview. *Cancer Res.* 30: 559-576 (1970).
2. Miller, E. C., Miller, J. A., and Hartman, H. A. *N*-Hydroxy-2-acetylaminofluorene: a metabolite of 2-acetylaminofluorene with increased carcinogenic activity. *Cancer Res.* 21: 815-824 (1961).
3. Debaun, J. R., Rowley, J. Y., Miller, E. C., and Miller, J. A. Sulfotransferase activation of *N*-hydroxy-2-acetylaminofluorene in rodent livers susceptible and resistant to this carcinogen. *Proc. Soc. Exptl. Biol. Med.* 129: 268-273 (1968).
4. Ivery, C. C. Metabolic activation of *N*-hydroxy compounds by conjugation. *Xenobiotica* 1: 387-398 (1971).
5. Lotlickar, P. D. Acylation of carcinogenic aromatic hydroxamic acids by acetyl-co-A and carbamoyl phosphate to form reactive esters. *Xenobiotica* 1: 543-544 (1971).
6. King, C. M. Mechanism of reaction, tissue distribution and inhibition of arylhydroxamic acid acyltransferase. *Cancer Res.* 34: 1503-1515 (1974).
7. Beland, F. A., Allaben, W. T., and Evans, F. E. Acyltransferase-mediated binding of *N*-hydroxy arylamides to nucleic acids. *Cancer Res.* 40: 834-840 (1980).
8. Miller, E. C., Miller, J. A., and Enomoto, M. The comparative carcinogenicities of 2-acetylaminofluorene and its *N*-hydroxymetabolite in mice, hamsters and guinea pigs. *Cancer Res.* 24: 2018-2031 (1964).
9. Schut, H. A. J., and Thorgeirsson, S. S. *In vitro* metabolism and mutagenic activation of 2-acetylaminofluorene by subcellular liver fractions of Cotton rats. *Cancer Res.* 38: 2501-2507 (1978).
10. Dyer, H. M., Kelly, M. G., and O'Gara, R. W. Lack of carcinogenic activity and metabolic fate of fluorenylacetamides in monkeys. *J. Natl. Cancer Inst.* 36: 305-322 (1966).
11. Reddy, T. V., Weisburger, E. K., and Thorgeirsson, S. S. Mutagenic activation of *N*-2-fluorenylacetamide and *N*-hydroxyfluorenylacetamide in subcellular fractions from X/Gf mice. *J. Natl. Cancer Inst.* 64: 1563-1569 (1980).
12. Michalopoulos, G., Russell, F., and Biles C. Primary cultures of rat hepatocytes on human fibroblasts. *In Vitro* 15: 796-806 (1979).
13. Michalopoulos, G., Strom, S. C., Kligerman, A. D., Irons, G. P., and Novicki, D. L. Mutagenesis induced by procarcinogens at the hypoxanthine-guanine phosphoribosyltransferase locus of human fibroblasts cocultured with rat hepatocytes. *Cancer Res.* 41: 1873-1878 (1981).
14. Strom, S., Kligerman, A. D., and Michalopoulos, G. Comparisons of the effects of carcinogens in mixed cultures of hepatocytes and human fibroblasts. *Carcinogenesis* 2: 709-715 (1981).
15. Strom, S. C., Jirtle, R. L., Jones, R. S., Novicki, D. L., Rosenberg, M. R., Novotny, A., Irons, G., McLain, J. R., and Michalopoulos, G. Isolation, culture and transplantation of human hepatocytes. *J. Natl. Cancer Inst.*, in press.
16. Howard, P. C., Casciano, D. A., Beland, F. A., and Shaddock, J. G., Jr. The binding of *N*-hydroxy-2-acetylaminofluorene to DNA and repair of the adducts in primary rat hepatocyte cultures. *Carcinogenesis* 2: 97-102 (1981).
17. Visser, A., and Westra, J. G. Partial persistency of 2-aminofluorene and *N*-acetylaminofluorene in rat liver DNA. *Carcinogenesis* 2: 737-740 (1981).
18. Meerman, J. H. N., Beland, F. A., and Mulder, G. J. Role of sulfation in the formation of DNA adducts from *N*-hydroxy-2-acetylaminofluorene in rat liver *in vitro*. Inhibition of *N*-acetylated aminofluorene adduct formation by pentachlorophenol. *Carcinogenesis* 2: 413-416 (1981).
19. Michalopoulos, G., Sattler, G. L., O'Conner, L., and Pitot, H. C. Unscheduled DNA synthesis induced by procarcinogens in suspensions and primary cultures of hepatocytes on collagen membranes. *Cancer Res.* 38: 1866-1871 (1978).
20. Williams, G. M. Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell cultures. *Cancer Res.* 37: 1845-1851 (1977).
21. Yager, J. D., Jr., and Miller, J. A., Jr. DNA repair in primary cultures of rat hepatocytes. *Cancer Res.* 38: 4385-4394 (1978).
22. Dybing, E., von Bahr, C., Aune, T., Glaumann, H., Levitt, D. S., and Thorgeirsson, S. S. *In vitro* metabolism and activation of carcinogenic aromatic amines by subcellular fractions of human liver. *Cancer Res.* 39: 4206-4211 (1979).
23. Regan, J. D., and Setlow, R. B. Two forms of repair in the DNA human cells damaged by chemical carcinogens and mutagens. *Cancer Res.* 34: 3318-3325 (1974).
24. Aust, A. E., Falahee, K. J., Maher, V. M., and McCormick, J. J. Human cell-mediated benzo(a)pyrene cytotoxicity and mutagenicity in human diploid fibroblasts. *Cancer Res.* 40: 4070-4075 (1981).