Pleiotropic Drug Resistance in Hepatocytes Induced by Carcinogens Administered to Rats

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ABSTRACT

The effect of hepatocarcinogen administration in vivo on the induction of pleiotropic drug resistance was studied in primary monolayer cultures of adult rat hepatocytes using a cytotoxicity assay in vitro. Dietary 2-acetylaminofluorene, 3'-methyl-4-dimethylaminoazobenzene, aflatoxin B1, ethionine, and diethylaminoethylamine rapidly induced resistance to doses of Adriamycin, methotrexate, cycloheximide, and aflatoxin B1 which were cytotoxic to normal hepatocytes from untreated rats. Up to 95% of some hepatocyte preparations became drug resistant before any new hepatocyte phenotypes could proliferate. Drug resistance was measured at 24 h after initiation of 2-acetylaminofluorene feeding and remained stable throughout the 16 wk of carcinogen exposure. When limited carcinogen exposure was followed by a return to a basal non-carcinogen-containing diet for many months, the hepatocytes in the resultant hepatocellular carcinomas also displayed pleiotropic drug resistance, and the cells of the peritumorous liver did so to a lesser extent. Drug resistance was not induced by chronic administration of the tumor promoters phenobarbital, choline-deficient diet, phorbol, nor with 2,3,7,8-tetrachlorodibenzo-p-dioxin, but was induced to a variable extent by three hepatotoxins (ethanol, methotrexate, carbon tetrachloride). Whereas the early appearing drug resistance appears to be an adaptation of the liver to the presence of a toxic carcinogen, the late resistance which does not disappear after withdrawal of the inducing carcinogen may be a constitutive characteristic of chemically induced hepatocellular carcinomas.

INTRODUCTION

The idea that the cells of an experimental animal which had been previously exposed to a toxin might be altered so as to be resistant to the damaging effects of a subsequent exposure to the same or a different toxin originated with the experiments of MacNider (1-4) and Hunter (5) using uranium salts and chloroform to study hepatic and renal toxicity. Subsequently, Haddow showed that, although most PAH carcinogens had a variety of toxic and growth-inhibitory properties in vivo, the tumors which they induced were relatively resistant to the toxic and growth-inhibitory effects of PAHs (6-9). He showed that growth-inhibitory and carcinogenic potencies of PAHs were parallel, and that carcinogens inhibited growth in the order: normal tissues > spontaneously occurring tumors > chemically induced tumors (10). This led to the hypothesis of "cellular inhibition and the origin of cancer" (11), in which it was proposed that, since many chemical carcinogens were toxins, the neoplastic cells that developed in this toxic environment must have acquired a resistance to the toxicity of the inducing carcinogen. The relative resistance of tumor cells compared to normal cells to growth inhibition by toxins, carcinogenic or otherwise, was noted by several authors (12-14). Acquisition of this relative resistance to the cytotoxic and growth-inhibitory action of carcinogens was observed in both chemically (15-18) and virally (19-21) transformed cells. The paradox, that chemical carcinogens are toxic to normal cells and appear to induce a relative inhibition of normal cell growth yet induce and stimulate tumor cell growth, has been previously reviewed (22-25).

Two types of carcinogen-induced resistance have been discerned. The first is characterized by resistance to cytotoxicity, assessed as resistance to cell necrosis or biochemical damage in vivo (26, 27) or resistance to cell death in vitro (28-31). The second is resistance to the antiproliferative action of a carcinogen. This has been observed in vivo (32-35) and in vitro (36, 37) as the ability of carcinogen-altered cells to proliferate under conditions in which a carcinogen can inhibit the proliferation of nonresistant normal cells. Carcinogen-induced resistance to both toxicity and mitoinhibition has been studied most extensively in rodent liver. Many carcinogens are also cell toxins, and one acute effect of many hepatocarcinogens is an inhibition of hepatocyte proliferation (38, 39). However, after chronic administration of a hepatocarcinogen, foci and nodules of altered hepatocytes grow despite the continuing presence of a toxic carcinogen. Such foci and, subsequently, nodules have apparently developed a resistance to the antiproliferative actions of the hepatocarcinogens that induced their growth. In addition to the antiproliferative effects, carcinogen-induced liver nodules are also relatively resistant to the hepatotoxic and necrogenic actions of both the inducing and different hepatocarcinogens in vivo (26, 28).

HCC is one of the most common cancers to afflict humans, due to its high frequency in Southeast Asia and sub-Saharan Africa (40). The growth of HCC in humans is particularly resistant to the growth-inhibitory effects of both ionizing radiation and most known cancer chemotherapeutic agents (41, 42). The resistance of human HCC to the toxic actions of most agents used for cancer therapy and the resistance of carcinogen-altered rat hepatocytes to cytotoxicity thus appear to have some similarities. In initial experiments, I examined hepatocytes in vitro from rats treated with the hepatocarcinogen AAF in vivo and found that, compared to normal hepatocytes, they were resistant to the cytotoxic effects of many toxic compounds, including several agents that are used in cancer chemotherapy such as Adriamycin (30, 43). The present paper extends the previous work, by examining hepatocytes in vitro from rats administered several hepatocarcinogens, in order to determine: (a) whether several other hepatocarcinogens can induce resistance to toxicity by chemotherapeutic agents and other cell toxins in hepatocytes; (b) how early in carcinogen administration resistance occurs and how stable it is; and (c) whether carcinogen-induced resistance is a general nonspecific adaptation to the presence of a toxic carcinogen, or whether it is a specific property of HCC. For the last goal, HCCs were examined many months after withdrawal of the inducing carcinogen in order to avoid the confounding issue of alterations in hepato-
tocytocytes which might be an adaptation to the chronic presence of a toxic carcinogen.

MATERIALS AND METHODS

Animals. Young adult male F344 rats, 180- to 200-g weight (Simonsen Laboratories, Inc., Gilroy, CA), were fed either a basal, high casein diet (Bio-Mix 101; Bio-Serv, Inc., Frenchtown, NJ) or a basal diet supplemented with either AAF (0.02%, w/w), M-DAB (0.06%, w/w), ethionine (0.25% w/w), aflatoxin B1 (nominal 1 ppm, gift of MRC Toxicology Unit, Carshalton, United Kingdom), PB (0.05%, w/w), or a CD diet (Dyets, Inc., Bethlehem, PA). The ethanol diet was a 150-cal liquid diet (Bio-Mix 711; Bio-Serv, Inc., Frenchtown, NJ). Animals were maintained on a 12-h light cycle in our small animal vivarium with unlimited access to food and water. Some rats were administered drinking water with DEN at 80 ppm (44). Three regimens were used for the production of HCCs, all using limited carcinogen exposure. (a) For the regimen of Solt and Farber (45) rats were given injections of DEN (200 mg/kg i.p. in 0.9% NaCl solution) and 2 wk later were placed on an AAF-containing diet for 2 wk. After 1 wk on the AAF diet, the rats were subjected to a PH. (b) Rats were given DEN (30 mg/kg) 24 h after a PH and then fed a PB-containing diet (46). (c) Rats were fed AAF for 12 wk and then returned to a basal diet (47).

Chemicals. AAF, DEN, ethionine, 2-aminofluorene, 2-nitrofluorene, 2-amino-9-fluorenone, fluorene, and anthracene were obtained from Aldrich Chemical Co. (Milwaukee, WI); M-DAB, from Ruge Chemical Co., Inc. (Irvington, NJ); aflatoxin B1, and cycloheximide, from Sigma Chemical Co. (St. Louis, MO); phenobarbital, from Mallinckrodt, Inc. (St. Louis, MO); PMA and phorbol, from Chemicals for Cancer Research (Eden Prairie, MN); methotrexate, from Lederle Laboratories, Division of American Cyanamid Co. (Pearl River, NY); Adriamycin was a gift of Adria Laboratories (Columbus, OH); carbon tetrachloride (CCL4), from Eastman Kodak Co. (Rochester, NY) and TCDD, from Dow Chemical Co. (Midland, MI).

Chemical Usage. DEN was diluted in tap water for chronic administration and in 0.9% NaCl solution for injections. TCDD was diluted in p-dioxane and injected s.c. as 1.4 µg/kg every 7 days (48). PMA and phorbol were given i.p. in 0.9% NaCl solution as 200 µg per injection every 7 days (49); CCL4 was diluted 1:1 in corn oil and given as 0.3 ml s.c. twice weekly (for 52 weeks). Methotrexate was given in drinking water at 2.5 mg/liter. For cytotoxicity assays in vitro, Adriamycin, methotrexate, and cycloheximide were freshly dissolved in 0.9% NaCl solution and used at final concentrations of 1.8 x 10^-4 M, 5.4 x 10^-3 M, and 7.0 x 10^-2 M, respectively. Aflatoxin B1 was dissolved in dimethyl sulfoxide, its concentration being determined spectrophotometrically and stored as 1 x 10^-4 M at 4°C. Immediately prior to use, it was diluted to 1 x 10^-4 M final concentration using Leibowitz Medium 15.

Primary Monolayer Hepatocyte Cultures. Hepatocyte suspensions were prepared by the proteolytic enzyme perfusion technique (50). Cell suspensions were passed through sterile gauze filters to remove undissociated tissue fragments, and viability was assessed by trypan blue exclusion. Hepatocytes (viability, >85%) were plated at 1 x 10^6 viable cells per plastic screw-cap tissue culture flask (Falcon Plastics, Oxnard, CA; 25-cm² surface area) in 4 ml of Leibowitz Medium 15 (Gibco, Grand Island, NY) with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (3.5 mg/ml) and bovine serum albumin (2 mg/ml) supplemented with 10% fetal bovine serum, penicillin (100 µl/ml), and streptomycin (100 µg/ml). After a 3-h attachment period at 37°C in a water-saturated atmosphere (3.5 mg/ml) and bovine serum albumin (2 mg/ml) supplemented with 10% fetal bovine serum, penicillin (100 µl/ml), and streptomycin (100 µg/ml). After a 3-h attachment period at 37°C in a water-saturated atmosphere, the cells were washed 3 times in the above medium and then placed in 4 ml of fresh medium and serum with (experiment) or without (controls) cytotoxic drugs.

Quantitation of Cell Resistance. Monolayer cultures were incubated for 24 h with or without the test cytotoxins. The 4 test cytotoxins were chosen on the basis of previous studies (28-30) as being hepatotoxins with 4 completely different modes of action. Adriamycin was specifically included because of its use in the clinical treatment of hepatocellular carcinoma, although its activity is limited as judged by tumor responses and patient survival. The doses used are higher than in most in vitro clonogenic assays, since higher doses are needed to kill quiescent cells in this system (by contrast, 10^-4 M Adriamycin suffices to inhibit epidermal growth factor-induced DNA synthesis). At the end of the incubation, 0.8 ml of trypsin blue were added to the 4 ml of medium in each flask and incubated for 10 min at 37°C. The medium was then removed, and the number of viable nonstaining hepatocytes was counted exactly as described (30). The number of attached viable (resistant) hepatocytes in the experimental flasks was compared to the number of attached viable hepatocytes in the control flasks, and the ratio was expressed as the percentage of survival. Under these experimental conditions, >95% of the cells used were hepatocytes, and none of them proliferated during the 24 h of the cytotoxicity assay. Statistical analysis of the data was performed using Student's t test.

Histochemical Staining for γ-Glutamyltranspeptidase. Rats which were subjected to the regimen of Solt and Farber were sacrificed at intervals, and their livers were removed and slices were taken from the largest diameter of each liver lobe. A composite was made of all the slices from each lobe and frozen. Standard cryostat sections were prepared from each liver composite, and 2 contiguous sections were stained for γ-GT exactly as described (51). The slides which were stained for γ-GT were examined under a dissecting microscope. The number of positively staining areas per section was counted and expressed as the number of foci/cm² of cross-section of the composite. The surface area of the composite was calculated by tracing the outline of the composite on graph paper, which was then weighed. The area of the weighed paper was calculated by reference to a standard curve, which was made by weighing squares of graph papers of known surface area.

RESULTS

AAF Exposure: Onset and Stability of Resistance. It was previously demonstrated that AAF feeding for 3 mo induced a pleiotropic drug resistance in the altered rat hepatocytes (30). In order to determine when drug resistance appears in carcinogen-fed rats, dietary AAF was administered and rats were sacrificed at intervals in order to obtain primary hepatocyte cultures. Cytotoxicity assays were performed on normal or AAF-altered hepatocytes using the 4 test toxins used previously, namely, Adriamycin, methotrexate, cycloheximide, and aflatoxin B1. Resistance was observed by 24 h of initiating AAF feeding and at all time points measured up to 16 wk (Fig. 1). The increase in resistance at 24 h was significantly different (P < 0.01) from the measurements on normal rats (Day 0) for all 4 test toxins. This difference persisted at wk 4 and wk 12, with

![Fig. 1. The development of pleiotropic drug resistance in primary monolayer cultures of hepatocytes after dietary administration of AAF. At each time point, primary monolayer cultures of rat hepatocytes were obtained, and cytotoxicity assays were performed. Each point represents the mean of results from 3 rats (3 experiments), 3 flasks per test drug per experiment; bars, SD. % Survival, percentage of attached, viable cells in the flasks containing the test drug compared to attached viable cells in flasks which did not contain the test drug (controls) after a 24-h incubation in vitro. The test drugs used were: Adriamycin, 1.8 x 10^-4 M (a); methotrexate, 5.4 x 10^-3 M (c); aflatoxin B1, 1.0 x 10^-4 M (c); or cycloheximide, 7.0 x 10^-4 M (c).](https://cancerres.aacrjournals.org)
CARCINOGEN-INDUCED DRUG RESISTANCE

Other Hepatocarcinogens. In order to determine whether the early induction of drug resistance was associated with the action of other hepatocarcinogens, aflatoxin B,

M-DAB, ethionine, and DEN were chronically administered to a group of rats at concentrations previously shown to be completely carcinogenic. Some rats from each group were sacrificed at intervals, and the resistant properties of their hepatocytes were examined in

primary monolayer cultures using the same battery of 4 test toxins (Fig. 3). Administration of aflatoxin B,

in the rat diet, one of the two main candidate factors together with hepatitis B virus in the etiology of human HCC, resulted in profound resistance to the cytotoxic effects of aflatoxin B,

and Adriamycin, as well as in resistance to methotrexate, but no resistance to cycloheximide (Fig. 3A). The aflatoxin B,

induced resistance occurred later than resistance induced by AAF feeding. The administration of M-DAB resulted in resistance to all 4 test toxins (Fig. 3B), although this was also slower than the immediate induction of resistance induced by AAF administration, since no resistance was found at 24 h (data not shown). Dietary ethionine also induced resistance to all 4 test toxins (Fig. 3C), resistance to cytotoxicity mediated by Adriamycin being the earliest and most profound, and resistance to the other three test toxins occurring moderately slowly. Administration of DEN in the drinking water had similar effects (Fig. 3D), with resistance to the 4 test toxins appearing at slightly different intervals for each toxin and Adriamycin resistance occurring early. In all of these regimens, resistance to the cytotoxic actions of Adriamycin was the most profound. In all regimens, no attempt was made to separately study nodular and nonnodular hepatocytes.

Regimen of Solt and Farber. The above 5 regimens of hepatocarcinogen administration involved chronic carcinogen exposure. The regimen of Solt and Farber was therefore chosen for investigation, since this involved a limited hepatocarcinogen administration (Fig. 4). Resistance was not found after a single DEN dose, which is also toxic (52), but appeared with the onset of AAF administration as noted in Fig. 1, and reached a peak on the day of the PH (measurements done a few hours after the PH). In contrast to 4-wk AAF exposure alone (Fig. 2B), however, resistance did not disappear with time after cessation of AAF exposure. A slight decrease in resistance was seen at the end of AAF feeding, but a return and persistence of pleiotropic drug resistance occurred, which lasted through 1 yr (Fig. 4A). Adriamycin resistance was the most profound in the early weeks. Induction of resistance with this regimen was compared with the development of foci staining for \( \gamma \)-GT (Fig. 4B). Induction of both \( \gamma \)-GT-positive foci and resistance occurred maximally at the time of the AAF administration (Fig. 4A and B). However, the number of \( \gamma \)-GT-positive foci then underwent a decline as noted elsewhere (53) and increased slowly thereafter, whereas resistance stayed at high levels after a transient decrease at 4 wk when dietary AAF was withdrawn.

Nodules and Hepatocellular Carcinomas. The experiments described above concern drug resistance found in primary monolayer cultures of hepatocytes obtained from rat liver after portal vein perfusion of collagenase. The perfusate represents a mixture of both "normal" hepatocytes and those from enzyme-altered foci and nodules. The high proportion of resistant hepatocytes in some of these experiments suggested that both tumor precursor hepatocytes and nontumor precursor hepatocytes might participate in the adaptive resistance to the presence of a hepatocarcinogen. In order to investigate this further, the sequential regimen of AAF feeding and withdrawal was used (54) in order to obtain large nodules that could be macroscopically observed and removed. The nodular hepatocytes were
Carcinogen-Induced Drug Resistance

Table 1. Resistance induced in nodules and HCC, and by various chemicals.

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>Duration of treatment</th>
<th>No. of rats</th>
<th>Adriamycin (1.8 × 10⁻⁴ M)</th>
<th>Methotrexate (5.4 × 10⁻³ M)</th>
<th>Cycloheximide (7.0 × 10⁻³ M)</th>
<th>Aflatoxin B₁ (1.0 × 10⁻⁴ M)</th>
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<tbody>
<tr>
<td>a. AAF nodules</td>
<td>16 wk</td>
<td>4</td>
<td>95 ± 5&lt;sup&gt;5&lt;/sup&gt;</td>
<td>75 ± 8</td>
<td>58 ± 14</td>
<td>93 ± 6</td>
</tr>
<tr>
<td>b. AAF perinodular tissue&lt;sup&gt;6&lt;/sup&gt;</td>
<td>16 wk</td>
<td>4</td>
<td>72 ± 4</td>
<td>53 ± 6</td>
<td>45 ± 5</td>
<td>67 ± 8</td>
</tr>
<tr>
<td>c. AAF tumor</td>
<td>12 mo</td>
<td>3</td>
<td>91 ± 5</td>
<td>65 ± 7</td>
<td>60 ± 5</td>
<td>85 ± 9</td>
</tr>
<tr>
<td>d. DEN/PB tumor</td>
<td>15 mo</td>
<td>6</td>
<td>54 ± 12</td>
<td>38 ± 10</td>
<td>35 ± 4</td>
<td>45 ± 6</td>
</tr>
<tr>
<td>e. DEN/PB peritumor&lt;sup&gt;6&lt;/sup&gt;</td>
<td>15 mo</td>
<td>6</td>
<td>22 ± 6</td>
<td>15 ± 5</td>
<td>12 ± 2</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>f. Solt/Farber tumor</td>
<td>15 mo</td>
<td>4</td>
<td>82 ± 5</td>
<td>65 ± 13</td>
<td>58 ± 15</td>
<td>88 ± 12</td>
</tr>
<tr>
<td>g. Solt/Farber peritumor&lt;sup&gt;6&lt;/sup&gt;</td>
<td>15 mo</td>
<td>4</td>
<td>45 ± 9</td>
<td>31 ± 6</td>
<td>25 ± 8</td>
<td>48 ± 4</td>
</tr>
<tr>
<td>h. PB</td>
<td>15 mo</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>i. CD</td>
<td>3 mo</td>
<td>3</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>j. TCDD</td>
<td>3 mo</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>k. PMA, phorbol</td>
<td>3 mo</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>l. Ethanol</td>
<td>12 mo</td>
<td>3</td>
<td>30 ± 12</td>
<td>9 ± 6</td>
<td>74 ± 10</td>
<td>49 ± 7</td>
</tr>
<tr>
<td>m. CCL</td>
<td>3 mo</td>
<td>2</td>
<td>15 ± 7</td>
<td>7 ± 4</td>
<td>12 ± 3</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>n. Methotrexate</td>
<td>3 mo</td>
<td>2</td>
<td>0</td>
<td>26 ± 4</td>
<td>0</td>
<td>11 ± 6</td>
</tr>
<tr>
<td>o. Age controls (no treatment)</td>
<td>12 mo</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<sup>a</sup> See "Materials and Methods."  
<sup>b</sup> Results of cytotoxicity assay using fresh preparations of hepatocytes in primary monolayer culture. The percentage of survival is after incubation with (experiment) or without (controls) test toxins for 24 h, as in Fig. 1.  
<sup>c</sup> Mean ± SD.  
<sup>d</sup> Comparisons were made of the data between the pairs a and b, d and e, f and g, using Student's t test. The pairs each differed, P < 0.001.

Fig. 3. Pleiotropic drug resistance induced in rat hepatocytes by chronic dietary administration of complete hepatocarcinogens. At each time point, primary monolayer hepatocyte cultures were obtained, and cytotoxicity assays were performed. Results are from 2 rats per time point. For each drug, triplicate flasks were used. Points, mean; bars, SD. Each point is the mean of 3 flasks per rat; 2 rats were used for each time point.

Fig. 4. Pleiotropic drug resistance and γ-GT-positive foci induced by limited carcinogen exposure by the regimen of Solt and Farber (45). Rats were administered the hepatocarcinogenic regimen as detailed in "Materials and Methods." In A, at intervals, primary monolayer cultures of rat hepatocytes were obtained, and a cytotoxicity assay was performed. Points, mean of 3 flasks per drug per rat (2 rats); bars, SD. The test drugs used were: Adriamycin, 1.8 × 10⁻⁴ M (●); methotrexate, 5.4 × 10⁻³ M (□); aflatoxin B₁, 1.0 × 10⁻⁴ M (▲); cycloheximide, 7.0 × 10⁻³ M (▲); Adriamycin, 1.0 × 10⁻⁴ M (▲); or cycloheximide, 7.0 × 10⁻³ M (▲). In B, at intervals, 2 rats per time point were sacrificed, and a composite of sections from each liver was made and stained histochemically for γ-GT ("Materials and Methods"). Each point, results for 2 composite sections per liver from 2 rats.
of a carcinogen, three regimens were used for the chemical production of hepatocellular carcinomas which used limited carcinogen exposure several months prior to the cytotoxicity assay ("Materials and Methods"). Table 1 shows the drug-resistant properties of hepatocytes from tumors produced by 12 wk of AAF feeding 1 yr previously. A profound degree of resistance was observed despite the absence of any carcinogen for 9 mo. In addition to the hepatocellular carcinomas, the livers affected by this regimen had multinoularity throughout their volume; consequently, peritumorous and nonnodular liver could not be removed and separately evaluated with any confidence. However, the regimen of DEN and phenobarbital administration (46) produced tumors after 15 mo that were relatively well localized, and nontumorous liver could be removed and separately studied after the liver perfusion. In these tumors, pleiotropic drug resistance was observed which was considerably greater \((P < 0.001)\) than the resistance found in the peritumorous hepatocytes (Table 1), in contrast to the finding in the perinodular hepatocytes after AAF feeding. Comparable results were obtained with tumors and peritumorous tissue produced by the regimen of Solt and Farber (45) (Table 1) in which resistance in tumor hepatocytes was significantly greater \((P < 0.001)\) than in peritumorous hepatocytes. Dietary administration of several compounds structurally related to AAF was also examined using each analogue as 0.02% diet (Table 2). Some resistance was induced by 2-aminofluorene which is also a hepatocarcinogen (57), but no resistance was found after administration of 2-nitrofluorene, 2-amino-9-fluorenone, fluorene, or anthrachene.

Promoters and Hepatotoxins. The effects of 4 agents with tumor-promoting activity were examined. Neither phenobarbital, a choline-deficient diet, TCDD, phorbol (hepatic tumor promoters), nor PMA alone produced significant resistance to the 4 test hepatotoxins (Table 1). However, ethanol, a hepatotoxin which is not thought to have hepatocarcinogenic properties in the rat, produced a considerable degree of resistance, although no nodules or tumors were found (Table 1). Two other hepatotoxins, methotrexate and carbon tetrachloride, were examined, and low levels of resistance were observed compared to age-matched controls (Table 1). Carbon tetrachloride is a hepatotoxin and hepatocarcinogen in the rat (55), and the level of resistance that it induced was low compared to the 5 complete hepatocarcinogens in Figs. 1 to 4. Methotrexate is a cancer chemotherapeutic agent, but has been widely used to chronically treat human psoriasis and has been shown to cause hepatic cirrhosis, fibrosis, and fatty liver (56). It induced resistance to cytotoxicity mediated by itself and to a lesser extent to aflatoxin B1.

**DISCUSSION**

Four structurally unrelated toxins with different mechanisms of action were used in vitro to probe the resistant properties acquired by hepatocytes from rats treated in vivo with various hepatocarcinogens. Chronic administration of 5 complete hepatocarcinogens led to the rapid induction of pleiotropic drug resistance in a large percentage of the hepatocytes. The time of induction and level of resistance differed for each carcinogen. For AAF, resistance was found at 24 h after carcinogen feeding began. This is likely to be too early an event to be explained by the selection or proliferation of resistant new cell types, and most probably represents an early adaptive change by the hepatocytes in response to the presence of a carcinogen. Since the early induction of resistance involves such a high proportion of the hepatocytes, it may not be a particular characteristic of the cancer progenitor cells alone. Of several AAF analogues (Table 2), resistance was only induced by 2-aminofluorene which also has some hepatocarcinogenic action (57).

In an attempt to distinguish hepatotoxic from hepatocarcinogenic influences on the induction of pleiotropic drug resistance, two approaches were taken. In the first approach, limited carcinogen exposure regimens were used, which resulted in hepatocellular carcinomas many months later. When resistance was investigated in the resultant tumors (Table 1), pleiotropic drug resistance was observed for HCC produced by all 3 regimens. The DEN and phenobarbital regimen induced the least amount of drug resistance. In all 3 regimens, hepatotoxicity was presumably not a factor at the time of the assay, since the cytotoxicity assay was performed many months after the last exposure of the rat to any hepatotoxin or hepatocarcinogen. In a second approach, hepatocytes from nodular and perinodular tissues and from tumorous and peritumorous tissues were compared (Table 1). Tumors produced by DEN and phenobarbital and by the regimen of Solt and Farber contained hepatocytes that were more drug resistant than hepatocytes from the peritumorous tissues. Peritumorous tissues could not be removed from tumors produced by 12 wk of AAF exposure with any confidence, due to the presence of multiple nodules throughout the liver. For this reason, a cyclical regimen designed to produce macroscopic nodules (54) was used. Hepatocytes from AAF nodules and from perinodular tissues both displayed pleiotropic drug resistance (Table 1). In this experiment, contamination of perinodular tissue by micronodular hepatocytes could not be excluded. Minimal resistance was noted after the promoting agents phenobarbital and a choline-deficient diet (Table 1), and no resistance was measurable after TCDD, phorbol, or PMA (Table 1). PMA, however, is not known to be a tumor promoter in the liver, although phorbol is (49). Among the 3 tested hepatotoxins, ethanol appeared to induce considerable resistance; methotrexate appeared only to induce resistance to methotrexate itself, and some resistance was noted after carbon tetrachloride, which also has hepatocarcinogenic properties (55).

Two types of resistance induced by hepatocarcinogens appeared to be distinguishable from these experiments. (a) Drug

<table>
<thead>
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<th>Table 2 Resistance induced by AAF and analogues</th>
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<tr>
<td><strong>Analogue</strong></td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td>AAF</td>
</tr>
<tr>
<td>2-Aminofluorene</td>
</tr>
<tr>
<td>2-Nitrofluorene</td>
</tr>
<tr>
<td>2-Amino-9-fluorenone</td>
</tr>
<tr>
<td>Flurene</td>
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<td>Anthrachene</td>
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* Analogue were administered as 0.02% (w/w) in diet.

* The percentage of survival is the result of the cytotoxicity assay of hepatocytes in primary monolayer culture as described for Fig. 1.

* Mean ± SD.
resistance in most hepatocytes was consequent on chronic carcinogen exposure. This may be a general adaptation to the chronically present hepatotoxin, since all 5 hepatocarcinogens that were administered are also hepatotoxins. Since such a high proportion of the hepatocytes are involved, this would seem unlikely to have a selective advantage to those cells that eventually develop into nodules. (b) Hepatocytes from HCCs displayed pleiotropic drug resistance which is probably independent of the presence of any toxin, since the tumors developed many months after the last exposure to any hepatotoxin or hepatocarcinogen. This could be due to the tumor phenotype per se or to the fact that HCCs contain a high proportion of proliferating cells, since proliferating cells from regenerating liver also have pleiotropic drug resistance (30, 58, 59).

The considerable level of drug resistance poses an interesting biological problem. Hepatocytes which have survived the chronic presence of hepatocarcinogens and hepatotoxins have acquired a large number of adaptations to toxic environments and a detoxification system which includes many elements, such as a decrease in the levels of cytochrome P-450 and an increase in glutathione, glutathione-S-transferases, UDP glucuronyl transferases, epoxide hydrolase, and DT-diaphorase (60). Multiple mechanisms have been postulated to underlie the resistance to cancer chemotherapeutic agents, mainly using sensitive and resistant tumor cell lines. Few data are available on the mechanisms involved in carcinogen-mediated resistance. Changes which are known to be associated with drug-resistant cell lines include decreased drug uptake, increased drug efflux, altered metabolism, and changes in drug-macromolecular binding. A glycoprotein which is increased in pleiotropically drug-resistant cells has also been described (61) and is associated with increased drug efflux and an amplified, over-expressed gene (62). Whether this one gene is the main explanation of resistance to drugs with different mechanisms is a matter of intense current experimentation. In AAF-altered resistant hepatocytes, altered drug efflux does not seem to occur in the case of Adriamycin, however (63). Although there are no data on drug resistance during the early stages of development of human HCC, if human HCCs are also induced by chronic exposure to chemical hepatocarcinogens such as mycotoxins, then they may also be constitutively resistant to the toxic effects of many of the currently known cytotoxic chemotherapeutic agents. If this view is correct, then clinical drug resistance in HCC, or possibly other chemically induced tumors, could be a characteristic of the tumor phenotype.

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CARCINOGEN-INDUCED DRUG RESISTANCE


Pleiotropic Drug Resistance in Hepatocytes Induced by Carcinogens Administered to Rats

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