Resistance to Hepatotoxins Acquired by Hepatocytes during Liver Regeneration

Esther Roberts, Miriam B. Ahluwalia, George Lee, Charlie Chan, D. S. R. Sarma, and Emmanuel Farber

ABSTRACT

The appearance of resistance to a number of hepatotoxins in primary cultures of hepatocytes prepared at various time intervals up to 2 weeks after partial hepatectomy is the major focus in this study. Resistance to the cytotoxic effect of aflatoxin B₁, 2-acetylaminofluorene, N-hydroxy-2-acetylaminofluorene, methotrexate, or methyl methanesulfonate shows a progressive increase until 48 hr and then returns to the resting level of susceptibility by 2 weeks. The genesis of mutagens from 2-acetylaminofluorene and aflatoxin B₁ by S-9 liver fractions shows a decrease from and return to control values after partial hepatectomy that parallels the resistance. The levels of total cellular cytochromes P-450 also decrease following partial hepatectomy and remain from 25 to 36% less than those of controls for at least 1 week. The glutathione and total soluble sulfhydryl ("glutathione") content increase following partial hepatectomy, and the pattern is consistent with a partial role for glutathione in the resistance phenomenon as it relates to 2-acetylaminofluorene. The possible relationship between resistance to the cytotoxic effects in vitro and the resistance to inhibition of cell proliferation in vivo during liver carcinogenesis is discussed.

INTRODUCTION

It has been shown in the past few years that the administration of many carcinogens, active on liver or other organs, when coupled with liver cell proliferation induces a small population of resistant hepatocytes in the liver (6, 35–37, 39). Such resistant hepatocytes can be stimulated to proliferate selectively by a mitogenic stimulus for liver in the presence of dietary 2-AAF sufficient to inhibit the proliferation of the majority of hepatocytes (25, 35, 36). In this way, hyperplastic nodules rapidly appear in the liver and metastasizing hepatocellular carcinoma has been found within such nodules (36). The existence of this material continuity among the carcinogen-induced resistant hepatocytes, a small subpopulation of hyperplastic nodules, and liver cancer indicates that some resistant hepatocytes may play an important role as an early precursor population in hepatocarcinogenesis under some experimental circumstances. Because of this and because hyperplastic nodules induced by carcinogens manifest a variety of properties related to the phenomenon of resistance to the cytotoxic and/or inhibitory effects of carcinogens on cell proliferation (3, 4, 11–13, 15, 19, 21, 22, 29, 30), we consider the fundamental nature of the resistance induced by a carcinogen to be an important subject for the study of mechanisms in the sequential analysis of liver cancer development (11).

Since cell proliferation is important both in initiation (6, 11) and in the genesis of hyperplastic nodules (11), it was considered appropriate to study some possible effects of cell proliferation per se on the resistance of hepatocytes without and with concomitant exposure to dietary 2-AAF before concentrating on the fundamental biochemical nature of the resistance phenomenon during carcinogenesis. The selection of an appropriate model for the study of mechanisms of resistance poses an immediate problem. Although some aspects can be studied in vivo, as reported previously for hyperplastic nodules (3, 12, 19, 22), an in vitro model would be highly desirable for studies on mechanism. However, in the absence of a reproducible system for long term culture of adult hepatocytes in which resistance to loss of both cell proliferation and cell integrity could be studied, a less satisfactory but still useful system was selected, the behavior of hepatocytes in primary cultures. This approach seemed particularly relevant since early in the course of this study Carr and Laishes (5) reported that, 24 hr after PH, isolated hepatocytes from normal rats show resistance to irreversible cell damage induced by AFB₁, cycloheximide, MTX, or Adriamycin. Previous studies by Laishes et al. (23, 24) and by Judah et al. (21) indicated the possible utility of this approach in studies on resistant hepatocytes. Hepatocytes isolated from hyperplastic nodules induced by some liver carcinogens showed resistance to concentrations of AFB, that ‘‘killed’’ hepatocytes from normal liver (24). Also, hepatocytes resistant to AFB, could be isolated from whole livers during aflatoxin-induced liver carcinogenesis (21).

The appearance of resistance to a number of hepatotoxins in primary cultures of hepatocytes prepared from animals at various intervals of time up to 2 weeks after PH and some possible mechanisms form the major focus in this communication.

MATERIALS AND METHODS

Animals. Male Fischer 344 rats (Charles River Breeding Laboratory, Inc., North Wilmington, Mass.) weighing 150 to 170 g were kept for 1 week on a basal semisynthetic high-protein (24%) diet (No. 101; BioServ, Inc., Frenchtown, N. J.). Water and food were available ad libitum. The animals were maintained on a 12-hr-light-12-hr-dark cycle. Some animals were fed the basal diet containing 0.02% 2-AAF for 1 or 2 weeks. In a few animals, hyperplastic nodules were induced by a standard 2-AAF regimen described previously (24).

Chemicals. Commercial chemicals were the purest grades available. They were obtained from the following sources: AFB₁ and 2-AAF from Aldrich Chemical Co., Milwaukee, Wis.; MTX (sodium injection) from Lederle Product Dept., Cyanamid of Canada, Montreal, Quebec, Canada; and DEM and collagenase type I from Sigma Chemical Co., St. Louis, Mo.

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Isolation of Hepatocytes. The method for enzymatic perfusion of the liver was slightly modified from that described previously (24). The perfusion with 0.05% collagenase type I was carried out at 37° for 8 to 12 min at a flow rate gradually decreasing from 40 ml/min to 10 ml/min. It was observed that livers following PH or exposure to dietary 2-AAF required longer enzymatic treatment than did normal liver to achieve the desired consistency of a thoroughly perfused liver. Thus, the termination of the treatment was based on subjective observations of the condition of the liver. There were no obvious differences in the viability of hepatocytes from normal or treated livers under these conditions of minor variability. The perfused liver was dissociated in an aliquot of the enzyme solution, then diluted with L-15 (Leibovitz) medium (Grand Island Biological Co., Grand Island, N. Y.), and centrifuged at 600 rpm for 4 min (at room temperature). The cells were resuspended in 50 ml L-15 and filtered through 2 layers of nylon mesh (ASTM-230-62). The cells were then centrifuged at 400 rpm, resuspended, and again centrifuged at 200 rpm. The final suspension upon which cell viability was determined was in L-15 medium supplemented with 10% fetal bovine serum (Flow Laboratories, Inc., Mississauga, Ontario, Canada) and 1% penicillin-streptomycin solution (penicillin, 10,000 units/ml; streptomycin, 10,000 µg/ml; Grand Island Biological Co.). The differential centrifugations produced cell suspensions consisting of at least 95% parenchymal cells. Based on 73 perfusions, the mean percentage of viability of the cell suspensions at the time of seeding was 79 ± 6 (S. D.).

Cell Cultures. Cell cultures were established by inoculation of 1 ml of the final cell suspension containing 1 to 1.2 × 10^6 viable cells (as determined by trypan blue exclusion test), into 25-cm² Falcon tissue culture flasks containing 3 ml of culture medium (L-15 supplemented as above). After an attachment period of 3 hr, the medium was removed and replaced by 4 ml fresh control medium or medium containing one of the test compounds at an appropriate concentration. The attachment efficiency at 3 hr (59 ± 4%) and percentage of survival at 48 hr (46 ± 3%) were not significantly different for hepatocytes isolated from control livers, from livers following partial hepatectomy, or from livers of animals exposed to dietary 2-AAF. Quantitation of Cytotoxicity. For each hepatotoxin, an initial dose response curve was determined using normal hepatocytes. A concentration which was toxic to normal hepatocytes (<10% survival at 48 hr) was selected. This concentration was then used on "experimental" (PH) hepatocytes. After 48 hr of incubation with the hepatotoxin or its vehicle, dimethyl sulfoxide, the cultures were washed with serum-free medium, and a solution containing 0.06% trypan blue was added to the flask for 5 min. The trypan blue was removed, and the number of viable cells were counted as described previously (24, 28). The mean number of cells per field was determined for control medium and with each of the test compounds. Each sample consisted of triplicate flasks, and ten 0.28-ml reticle fields, randomly chosen, were counted in each flask. The percentage of survival was calculated for each chemical by dividing the average number of cells per field remaining after treatment by the number of cells surviving without treatment (i.e., control medium is taken to represent 100% survival). The results are expressed as the mean ± S.E. of 3 to 5 experiments performed on different animals at different times.

Ames Mutagenesis Assay. The method used was that developed by Ames et al. (1). The tester strains, TA 100 and TA 1538, were obtained from Dr. Ames' laboratory. The liver homogenate fraction (S-9) was prepared as described (1). Protein determination was by the method of Lowry et al. (27). A dose response for each hepatotoxin was carried out using control S-9 from normal animals. A concentration which produced maximal mutagenesis without bacterial toxicity was used for all assays. Each S-9 preparation was tested at 3 or more concentrations to determine the level of optimum activity and to confirm the linearity of the response (Chart 1). Thus, consistently 50 and 100 µl of S-9 were assayed in triplicate for each sample. The number of revertants per plate was converted to revertants per mg protein (Chart 2). Larger volumes of S-9 were not used because of the prohibitive number of revertant colonies generated by normal livers and the decreased sensitivity incurred by counting such densely populated plates.

The results are expressed as the mean ± S.E. of samples obtained from each of as many as 15 animals.

Cytochrome P-450 Determination. At each time point following PH, animals were sacrificed, and livers were excised, weighed, homogenized, and assayed for cytochrome P-450 as described previously (4). Glutathione Experiments. Reduced and oxidized glutathione were measured by the method of Griffith (16). Livers were excised, plated, weighed, and homogenized in 5 volumes 1% picric acid at 0 to 4° with 3 passes of a teflon pestle. The homogenate was centrifuged for 15 min at 11,000 rpm in a Sorvall RC2, and the protein-free supernatant was frozen at −50°. For assay purposes, the supernatant was used undiluted (for oxidized glutathione) or diluted 80× with distilled water (for GSH).

GSH depletion was induced by the administration of DEM (60% in 0.9% NaCl or corn oil) at a dose of 0.1 ml/100 g body weight at 44 hr post PH. At 48 hr, some animals were sacrificed for GSH determinations. For these determinations, total "soluble sulphydryl group" was measured by the method of Eltman (10). Some animals were used for the preparation of isolated hepatocytes for measurement of resistance. The latter were perfused, plated, and exposed to the hepatotoxic...
agents in the continuing presence of 18 μM DEM (18, 37). In some experiments, glutathione, cysteine, N-acetylcysteine, or methionine were added in vitro during the exposure to the hepatotoxin.

RESULTS

Acquisition of Resistance after PH. PH results in the acquisition of the property of resistance to several carcinogens or cytoxins by a considerable proportion of the hepatocyte population. As indicated in Charts 3 to 7, hepatocytes isolated 48 hr after PH show a considerable decrease in their susceptibility to cell death induced by 2-AAF, N-OH-2-AAF, AFB₁, MTX, or MMS as compared to hepatocytes from control rats. The kinetics of acquisition of resistance varies somewhat from compound to compound. Resistance to each chemical appears by 18 hr (Chart 8). With AFB₁, maximum resistance is observed at 18 hr, with a smaller peak at 48 hr. Resistance to 2-AAF, N-OH-2-AAF, MMS, and MTX is maximal at 48 hr post PH.

The highest peak of resistance for each compound represents 43 to 74% of the viable hepatocyte population at that time. The cytotoxic effect is dose dependent for all chemicals as shown in Charts 3 to 7. In all cases, the high level of resistance subsides by 1 week following PH, and by 2 weeks control levels approaching zero are found (data not shown). Included for comparison in Charts 3 to 7 are data on the resistance of hepatocytes from hyperplastic nodules induced by 2-AAF. These cells show an intermediate degree of resistance with each of the 5 chemicals used.

Under these conditions using normal liver cells, PH seems to induce resistance which is nonselective, i.e., a resistant liver cell population is resistant to the direct acting hepatocarcinogen (MMS) as well as to those which require activation (AFB₁, etc.). Likewise, there is approximately equal resistance to N-OH-2-AAF, a compound which probably does not require a microsomal activation step, and to its precursor, 2-AAF, which does.

Do Hepatocytes Isolated from Livers following PH Acquire Resistance When Cell Proliferation is Inhibited? Animals were fed a diet containing 0.02% 2-AAF for 1 week prior to and 1 week following PH. Under these conditions, liver cell prolifer-
Different hepatotoxins when the animals were on basal diet. A, AFB$_1$, $10^{-7}$ M; O, 2-AAF, $2 \times 10^{-7}$ M; ●, N-OH-2-AAF, $2 \times 10^{-7}$ M; □, MTX, $5 \times 10^{-7}$ M; ○, MMS, $5 \times 10^{-7}$ M. The values shown are the mean of 4 to 9 animals per time point. Resistance to all chemicals is highly significant (p < 0.001) at 48 hr after PH.

Resistance to all chemicals except 2-AAF and MMS is highly significant (p < 0.001) at 48 hr and 1 week; resistance to MMS is significant (p < 0.05) at 48 hr post PH; resistance to 2-AAF is significant (p < 0.05) at 1 week.

The values shown are the mean of 3 to 10 animals per time point. Resistance to MMS, $5 \times 10^{-7}$ M, is almost completely inhibited (25, 35, 36). The 2-AAF diet alone for 7 days without PH induces very little if any resistance (Chart 9). Hepatocytes resistant to 2-AAF, N-OH-2-AAF, AFB$_1$, or MTX still appear at 18 hr or 24 hr after PH (Chart 9). Once induced by PH, a high level of resistance is maintained for 7 days, a period longer than for regenerating hepatocytes from animals on the control diet. In addition to this tendency to ‘‘turn off’’ their resistance, hepatocytes obtained after this regimen show a difference from their control counterparts in respect to MMS. Control cells following PH show the typical biphasic wave of resistance to $5 \times 10^{-7}$ M MMS, whereas cells from animals exposed to dietary 2-AAF show no resistance to the same concentration of MMS, except for one very small increase at 48 hr. However, at lower concentrations of MMS (Chart 7), the wide disparity between the control regenerating liver cells and cells from 2-AAF-exposed animals is not seen, both being clearly resistant compared to normal resting hepatocytes. It is noteworthy that the exposure to dietary 2-AAF for 1 week prior to performing PH did not increase the resistance of isolated hepatocytes to added 2-AAF, although such treatment did increase somewhat the resistance of the same cells to N-OH-2-AAF and AFB$_1$ (Charts 4 and 5). However, with continued exposure to the dietary 2-AAF, the cells do show more resistance to added 2-AAF than do comparable hepatocytes from control animals (data not presented).

Does the Post-PH Time Pattern of Acquisition of Resistance to 2-AAF and AFB, Correlate with Changes in (a) the Genesis of Bacterial Mutagens (Ames Test) or (b) the Levels of Microsomal Cytochrome P-450? Our data using the Ames bacterial system suggest that a decrease in microsomal activation does occur following PH and that it correlates with the onset of resistance of the whole cell to 2-AAF and AFB, (Chart 10). Thus, S-9 prepared from partially hepatectomized livers showed a decreased ability to generate revertants of TA 100 in the presence of AFB$_1$ and of TA 1538 in the presence of 2-AAF compared to the S-9 fraction from control resting liver. On the other hand, as anticipated, N-OH-2-AAF is not altered to a significantly lesser degree by S-9 from partially hepatectomized animals in spite of the observed resistance to N-OH-2-AAF of whole cells isolated from comparable animals.

Since 2-AAF and AFB$_1$, require cytochrome P-450-mediated metabolism for activation, it became of interest to determine whether the observed resistance might be associated with a decrease in the levels of total cytochromes P-450. The results indicate that the levels of cytochrome P-450 at different times after PH are significantly different at the earliest time point after PH 24 hr and remained so for at least 1 week (Table 1). The correlation between the time course of decrease in cytochrome
P-450 and resistance is by no means consistent throughout since the resistance is largely lost by 1 week; however, the level of cytochrome P-450 is still decreased at this time.

Can Changes in Glutathione Content Account for the Patterns of Resistance? Since glutathione has been suggested to play a role in one or more aspects of liver carcinogenesis with chemicals (7, 9, 13, 21, 29) and in the genesis of liver cell death (7, 18, 31, 38), it became of interest to explore a possible role for GSH in the resistance phenomenon. As seen in Table 2, the post-PH liver is associated with an increase in the concentration of glutathione as well as the total soluble sulfhydryl group. The magnitude of the increase is not very large, varying from 1.5- to 2.2-fold. The increase is seen at 24 and 48 hr and at 1 week. Depletion of the liver GSH levels by at least 68% by the administration of DEM was associated with an almost complete loss of resistance to 2-AAF by hepatocytes at 48 hr but with little if any effect with the other 4 hepatotoxins (Table 3). As indicated in Table 4, addition of DEM in vitro was necessary and perhaps even sufficient in order to show this loss of resistance. This finding is not unexpected since liver cells show a return to control levels of GSH on incubation in vitro following treatment of the animal with DEM (18, 38). In our studies, addition of DEM in vitro (18 jumol) was only slightly less effective in abolishing resistance to 2-AAF than was the combined in vivo-in vitro treatment. In 4 of 6 experiments, the decrease in resistance to 2-AAF seen in hepatocytes from DEM-treated animals was restored by the addition of GSH (1.8 to 18 jumol), L-methionine (0.5 to 3 mM), L-cysteine (0.5 to 3 mM), or N-acetyl-L-cysteine (0.5 to 3 mM) to the medium along with the DEM. Unfortunately, attempts to measure GSH levels in the small amounts of tissue at the end of the incubations were unsuccessful, and therefore no explanation for the inconsistency based on experimental data can be offered at this time.

**DISCUSSION**

It is evident from this study that hepatocytes develop a resistance to cytotoxic effects of 2-AAF, N-Oh-2-AAF, AFB1, MTX, and MMS after PH. This confirms in principle some of the conclusions of Carr and Laishes (5) who reported that resistance to AFB1, cycloheximide, Adriamycin, and MTX is present in liver cells at one time point, 24 hr, after PH. In our study, addition of DEM in vitro (18 jumol) was only slightly less effective in abolishing resistance to 2-AAF than was the combined in vivo-in vitro treatment. In 4 of 6 experiments, the decrease in resistance to 2-AAF seen in hepatocytes from DEM-treated animals was restored by the addition of GSH (1.8 to 18 jumol), L-methionine (0.5 to 3 mM), L-cysteine (0.5 to 3 mM), or N-acetyl-L-cysteine (0.5 to 3 mM) to the medium along with the DEM. Unfortunately, attempts to measure GSH levels in the small amounts of tissue at the end of the incubations were unsuccessful, and therefore no explanation for the inconsistency based on experimental data can be offered at this time.

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Since resistance was also exhibited by hepatocytes from rats that were exposed to a diet containing 0.02% 2-AAF for 1 week prior to and 1 week following PH, conditions under which cell proliferation is almost completely inhibited (25, 35), it would appear that cell proliferation or DNA synthesis is not a major factor for the acquisition of resistance to 2-AAF, N-OH-2-AAF, AFB1, or MTX.

At least 2 possible bases for the resistance were explored in this study, metabolic activation and increased protection by GSH. With respect to the first, the resistance is accompanied by a modest decrease in the ability of the S-9 fraction from each of the different liver preparations to generate mutagens from 2-AAF and from AFB1, and in the cellular levels of cytochrome P-450. The time frame for these changes is compatible with that for resistance. Our results with total cytochrome P-450 levels after PH are in general agreement with those of a
few other investigators who have studied the activities of this cytochrome system or of other components in the oxidative “drug-metabolizing” system in the microsomes (2, 14, 17, 32, 40). Aniline hydroxylation (17, 32), oxidative N-demethylation (2, 32, 40), and NADH:cytochrome c reductase (40) have each been shown to decrease following PH. Although the detailed time curves vary with each study, there is in general a modest decrease in several activities with the lowest levels from 20 to 60 hr following PH and return toward control values thereafter. These various results show that some decrease in microsomal oxidative metabolism could account, at least in part, for the peak of resistance seen by 48 hr post PH with 2-AAF and AFB1.

However, it appears unlikely that the levels of microsomal activation play the only role in the acquisition of resistance, since the latter is seen both with compounds that apparently do not require metabolism by the microsomal monoxygenase system as well as with compounds that do. MMS is not known to be dependent upon metabolism for its toxic effects and N-OH-2-AAF seems to be dependent upon esterification rather than microsomal activation for its conversion to a highly reactive and toxic metabolite.

With respect to the second possibility, alteration in the levels of GSH, there is a growing realization that this compound might play a major controlling factor in the removal or trapping of some reactive chemicals in the liver (7, 31). On the basis of the results of Neal et al. (29), it was anticipated that AFB1, toxicity might be most closely related to changes in GSH. However, in our study, alterations in GSH levels appear to relate to the resistance to only one hepatotoxin, 2-AAF. The reasons for this apparent discrepancy remain unknown. In this context, Levine (26) has reported recently that some protective effects of GSH in the liver might be mediated in part by its protection of one or more specific cytochromes P-450 from lipid peroxidation. Also, it has been reported that DEM, which reduces GSH concentrations in the liver, inhibits hepatic aryl hydrocarbon hydroxylase activity (8). Since varying the GSH levels had no obvious effect on the resistance to N-OH-2-AAF, the major site of action of GSH might be on one or more of the specific cytochromes P-450 or other enzymes involved in the metabolism of 2-AAF (20).

From the results reported by Carr and Laishes (5) and from this study, it is apparent that the biochemical events that follow PH involve a variety of aspects of cell metabolism. The 7 compounds used in these 2 studies differ considerably in their structure and in their metabolism. However, resistance is shown to each of them. This suggests that the biochemical programming appears to involve a package of properties rather than any single enzyme or pathway of enzymes. Also, these data emphasize again the inadequacy of our current understanding of the mechanisms of cell death. Since liver cell death is often the end stage of a multistep process (41), it is not surprising that no single proposed mechanism would appear to be adequate to explain its pathogenesis.

If the resistance measured in this model is a reflection of the resistance to inhibition of cell proliferation thought to be important in the genesis of hyperplastic nodules by differential inhibition (11), then hyperplastic nodules should be regularly produced by the selection procedure itself, i.e., dietary 2-AAF plus PH, without the need for an exposure to an initiating dose of another carcinogen. This has not been observed in many hundreds of animals studied over the past 4 years. It is quite possible that the 2 parameters used to measure resistance are indicative of 2 fundamentally different cellular responses to hepatotoxins. This question will remain unanswered until a much better understanding is obtained about the biochemical or metabolic basis of the 2 aspects of resistance.

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