Sex Differences in Cell Proliferation and N-Hydroxy-2-acetylaminofluorene Sulfotransferase Levels in Rat Liver during 2-Acetylaminofluorene Administration

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SUMMARY

This study was initiated in an effort to determine the mechanism responsible for the different susceptibilities of male and female rats to the induction of liver tumors by 2-acetylaminofluorene (AAF). Gain in body weight of male and female rats maintained on a diet containing 0.04% AAF for 8 weeks was inhibited 38 and 32%, respectively, when compared with animals on a control diet. Liver weights (g liver per 100 g body weight) of females remained the same during the 8-week period, while those of males decreased slightly between Weeks 3 and 8.

During the first 4 weeks of AAF administration, livers of male rats had 5 to 10 times the levels of N-hydroxy-AAF sulfotransferase activity as had livers of females. The absolute amount of sulfotransferase activity in livers of both male and female rats declined markedly and was approximately 10 to 30% of control values at the end of 2 weeks. In spite of the marked decrease in sulfotransferase activity, activity of the enzyme in livers of male rats remained 2 to 3 times that of females throughout the 8-week period.

The mitotic index and incorporation of tritiated thymidine in livers of female rats were low and constant throughout the 8-week period. In contrast, both thymidine incorporation and mitotic index in livers of male rats exhibited a marked increase after 3 weeks. Little or no change in content of liver RNA or DNA was observed in either male or female rats during the 8-week period.

INTRODUCTION

As early as 1941, Wilson et al. (26) reported a greater toxicity of AAF in male than in female rats. It was later confirmed by Skoryna and Webster (21) that AAF was more toxic to liver cells and produced a higher incidence of liver tumors in male, hooded rats than in female rats of the same strain. Other investigators have reported a similar sex difference in the effect of AAF in Sprague-Dawley rats (23). Recent studies (4, 25) have suggested that the reactive AAF-N-sulfate ester may be responsible for much of the binding of AAF in liver cells and that the level of N-hydroxy-AAF sulfotransferase may be correlated with the degree of hepatotoxicity and carcinogenicity. It is generally agreed that chemical carcinogenesis in liver cells is a multistage process (6). Laird and Barton (13) have divided the process of tumor induction by AAF in liver cells into 2 phases: (a) the 1st 4 to 8 weeks, during which no increase in cell number occurs and (b) the period after this in which there is continued cell proliferation. Numerous studies with other carcinogenic agents including urethan (17), UV radiation (18), and 3'-methyl-4-dimethylaminoazobenzene (7) suggest a requirement for a period of cell proliferation during tumor induction. The possible role of cell division in tumor induction was discussed recently by Warwick (24) and Farber (6).

In view of the apparent importance of cell proliferation in tumor induction, it was of interest to determine what role cell division might play in the different susceptibilities observed in male and female rats. Farber (5) observed proliferation of oval cells as early as Day 14 in livers of male rats receiving AAF, with development of extensive cell proliferation by Day 49. In contrast to male livers, however, livers of female rats appeared normal throughout the period studied. Teebor and Seidman (23) reported similar histological studies indicating no pathological changes of significance in livers of female rats throughout a 12-week period of AAF administration, in spite of the fact that a significant amount of carcinogen is bound to liver protein and RNA of female animals (11, 15) and that equal amounts of the carcinogen are bound to liver DNA in the 2 sexes (11). The purpose of this study was to quantitate and compare cell proliferation, nucleic acid content, and levels of N-hydroxy-AAF sulfotransferase activity in livers of male and female rats during administration of AAF p.o.

MATERIALS AND METHODS

Animals and Diet. Male and female Sprague-Dawley rats (180 to 200 g) were purchased from the Holtzman Co., Madison, Wis. All animals were fed the powdered grain diet previously described (10) for approximately 1 week before and throughout the experimental period. Diet containing 0.04% AAF was prepared by spreading the powdered diet in a large pan and applying the AAF dissolved in acetone over the surface with a pipet. After complete evaporation of acetone, the diet was thoroughly mixed.

Compounds. Thymidine-methyl-³H (8.07 Ci/mmmole) was
N-Hydroxy-AAF Sulfotransferase in Liver

N-Hydroxy-AAF sulfotransferase was purchased from New England Nuclear, Boston Mass. Each animal received 0.75 μCi thymidine-methyl-3H per g body weight and 3 mg colchicine in 0.9% NaCl solution i.p. 3 hr before it was sacrificed. AAF was synthesized by the method of Ray and Geiser (19).

**Determination of Mitotic Index.** Portions of each liver were fixed in neutral 10% formalin, cut in 5-μm sections, and stained with hematoxylin and eosin. For determination of mitotic index, sections were examined under oil immersion, and all hepatocytes with condensed chromosomes and dispersed nuclear membranes were scored as mitotic figures, regardless of phase of mitosis. A total of 2000 hepatic cells were counted for each animal.

**Nucleic Acid Determinations.** Nucleic acids were precipitated from a 25% liver homogenate by addition of perchloric acid (0°) to a final concentration of 0.2 N. The precipitate was washed twice with cold 0.2 N perchloric acid and digested in 0.3 N KOH at 37° for 1 hr. We made quantitative determinations of DNA on aliquots of the digest by the Ceriotti procedure (2). The digest was cooled to 0° and adjusted to a concentration of 0.5 N perchloric acid. After the removal of the DNA precipitate, RNA was determined in the supernatant by absorbance at 260 nm. The precipitate was washed with cold 0.5 N perchloric acid and digested in 0.5 N perchloric acid at 70° for 15 min. DNA content of the supernatant was determined by the method of Burton (1), and radioactivity was determined by liquid scintillation counting in Triton X fluor. Counting efficiency was determined by the addition of internal standard.

**Measurement of N-Hydroxy-AAF Sulfotransferase Activity.** N-Hydroxy-AAF sulfotransferase activity was determined in 105,000 × g supernatant of rat liver homogenate by the procedure in which the unstable AAF-N-sulfate that is formed is trapped by reaction with methionine (3, 9).

**RESULTS**

**Body and Liver Weight Gain.** Body weights of male and female rats on control and AAF-containing diets over the 8-week period studied are presented in Chart 1. Both male and female rats maintained on 0.04% AAF continued to gain weight over the 8-week period, although the rate of weight gain was less than normal. Compared with that of control animals of the same sex at the end of 8 weeks, the weight gain in male and female rats on 0.04% AAF was inhibited 38 and 32%, respectively. The reduced weight gain of animals on diets containing AAF probably was due both to the toxic effects of the carcinogen and to the fact that the animals ate less of the diet containing AAF.

Liver weights, expressed as g liver per 100 g body weight, to normalize individual differences in animal size, are shown in Chart 2. Compared with controls at Week 0, liver weights of female animals did not change significantly during the 8-week period. Livers of male animals exhibited a slight increase in weight during the 1st 2 weeks, followed by a decreased weight between Weeks 3 and 8.

**RNA and DNA Content.** When expressed as mg RNA per g liver, the liver RNA content of both male and female rats decreased during the 1st 3 weeks of AAF administration. However, when the results were expressed as mg liver RNA per 100 g body weight (Chart 3), the decrease was less pronounced. These results suggest that the increased liver weight observed during the 1st 2 weeks was probably due to changes in water or lipid content. Although RNA content of livers in both males and females was significantly lower after 2 weeks of AAF administration, livers of rats fed control powdered diet also had RNA contents significantly lower than those sacrificed at Week 0.

DNA content is also expressed as mg liver DNA per 100 g body weight (Chart 3). In contrast to the study of Schneider et al. (22) in which a marked increase in DNA content was observed during the 3rd week of 3'-methyl-4-dimethylaminoazobenzene administration, we found no significant change in liver DNA content in either male or female animals during the 8-week administration of AAF (Chart 3). Our results agree with the findings of others (22) that DNA content was not significantly increased during AAF administration.

**Chart 1.** Total body weight of Sprague-Dawley rats during AAF administration. Animals were fed control powdered grain diet or diet containing 0.04% AAF ad libitum. The following body weights, determined weekly, are shown: ○, male rats on a control diet; •, males given 0.04% AAF; ●, females given a control diet; and ♠, females given 0.04% AAF.

**Chart 2.** The liver weights of male (○) and female (●) rats that were maintained on a diet containing 0.04% AAF were determined at the time of sacrifice. Values shown are the mean of 4 animals ± S.E.
Chart 3. Liver nucleic acid content of rats on a diet containing 0.04% AAF. Values are expressed as mg total liver nucleic acid per 100 g body weight to normalize differences in size of individual animals. Nucleic acid content of male (○) and female (●) livers was determined as described in "Materials and Methods." Values represent the mean of 4 animals ± S.E.

with those of Laird and Barton (13), who reported a lag period of 7 to 8 weeks before significant increases in liver RNA or total liver cells were observed in male rats fed AAF.

Liver N-Hydroxy-AAF Sulfotransferase Activities. We previously reported that, after 3 days on a diet containing 0.04% AAF, liver N-hydroxy-AAF sulfotransferase activity of male rats was only 41% of that of animals on control diet, whereas no depression in activity of the enzyme was observed in females (8). Assays of livers of rats fed 0.04% AAF for 8 weeks revealed a marked drop in the level of N-hydroxy-AAF sulfotransferase activity (Chart 4). By the end of the 1st week on 0.04% AAF, N-hydroxy-AAF sulfotransferase activity in livers of male rats was depressed 50 to 70%, and the activity in livers of female rats was depressed 75 to 80%. Sulfotransferase activity in livers of male rats remained at 10 to 30% of control values from Weeks 2 to 8. Activity in livers of female rats, after falling to 10 to 20% of normal at Weeks 2 to 3, began to slowly return toward normal but remained at 35 to 40% of normal at Weeks 4 to 8. The data shown on Chart 4 were confirmed in a 2nd experiment in which additional groups of male and female rats were fed 0.04% AAF for 6 weeks. The ratio of activities of N-hydroxy-AAF sulfotransferase in male and female rat livers varied from 5 to 10 during the 1st 3 weeks of AAF feeding, then decreased to 2.5 from Weeks 4 to 8.

Thymidine Incorporation and Cell Proliferation. Incorporation of tritiated thymidine into liver DNA by male and female animals was determined at weekly intervals (Chart 5). During the 1st 2 weeks of AAF administration, incorporation of tritiated thymidine in both male and female rats did not change significantly. After Week 2 however, there was a marked increase in thymidine incorporation by livers from males. In contrast, thymidine incorporation by livers of female rats did not change significantly over the 8-week period.

Thymidine incorporation was paralleled by changes in the mitotic index (Chart 6). The mitotic index of livers from females remained very low (0 to 1/1000 cells) throughout the 8-week period. In males, the mitotic index of liver remained low (<1/1000 cells) during the 1st 2 weeks, after which a marked increase in mitotic figures occurred.
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A number of studies have suggested that this proliferation plays a role in carcinogenesis by chemicals (7, 14, 17, 24). The absence of liver cell proliferation in female rats fed AAF is probably due to the lower hepatotoxicity of AAF. Present evidence indicates that the hepatocarcinogenicity and the hepatotoxicity of AAF are each markedly dependent upon testosterone (3, 16, 20) which, in turn, may influence levels of N-hydroxy-AAF sulfotransferase activity in liver (3). AAF-N-sulfate has been proposed to be a key metabolite in inducing acute hepatotoxicity (massive periportal necrosis) and hepatocarcinogenicity in male rats (4, 25). Our data on the assays of rat liver for N-hydroxy-AAF sulfotransferase activity during 8 weeks of feeding AAF are consistent with this proposal. On the other hand, AAF does produce some deleterious effects in livers of female rats. This has been demonstrated by a decreased ability of livers of female rats to respond to enzyme induction (23) and to partial hepatectomy (12) after AAF administration.

We feel that an understanding of the mechanism of the differential response of livers of male and female rats to AAF will be most helpful in elucidating the mechanism of hepatocarcinogenesis. Comparison of the effects of the carcinogen in the 2 sexes should allow those effects that are incidental to be discerned from those that are required for the neoplastic transformation.

DISCUSSION

Although the mechanism responsible for different susceptibilities of liver to tumor induction by AAF in male and female Sprague-Dawley rats is unknown, it has generally been assumed to be correlated with differences in hepatotoxicity in the 2 sexes. Hepatotoxicity leads to cellular proliferation, and a number of studies have suggested that this proliferation plays an essential role in carcinogenesis by chemicals (7, 14, 17, 24). Quantitative data on the proliferative response of livers of male and female rats during continuous feeding of AAF have not been available. Our results indicate that there was no significant change in the extent of incorporation of thymidine-3H into liver DNA or in the mitotic index of liver during the first 2 weeks of administration of AAF to male and female rats. By the 3rd week, however, and thereafter for the 8-week period studied, thymidine-3H incorporation and mitotic index were significantly increased in livers of male rats but not in livers of female rats fed AAF. These data confirm the earlier histological observations made by Farber (5). Thus, the lack of cell proliferation seems to be a factor involved in the resistance of female rats to hepatocarcinogenesis by AAF.

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REFERENCES

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Chart 6. Liver cell proliferation in male (○) and female (•) rats maintained on 0.04% AAF. Experimental conditions were the same as in Chart 5. Mitotic index was determined as described in "Materials and Methods." Values shown are the mean of 4 animals ± S.E.


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