Effects of Sex Difference and Dietary Protein Level on the Binding of Aflatoxin B\textsubscript{1} to Rat Liver Chromatin Proteins \textit{in Vivo}\textsuperscript{1}

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ABSTRACT

The risk of aflatoxin-induced hepatocarcinoma is greater in males than in females and is similarly higher in animals fed 20% casein diets than in those fed 5%. In this study, groups of male and female F344 rats were fed either a 5 or 20% casein diet for 6 weeks. Two hr after a 1-mg/kg i.p. dose of \([\text{3H}]\) aflatoxin B\textsubscript{1}, animals were killed and four protein fractions were sequentially extracted from the liver chromatin of each. Within each treatment group, aflatoxin binding to nonhistones was greater than to histones, both before and after dialysis. Comparing treatments, the higher-risk factors were associated with a greater liver content of aflatoxin as well as with increased binding of both nondialyzable and dialyzable aflatoxin to the various fractions. The high degree of correlation between total liver content and adduct formation implicates the former as a major determinant of the latter. Also associated with the higher-risk factors was a shift in the distribution of dialyzable aflatoxin toward greater adduct formation with one of the nonhistone fractions, suggesting the possibility of a role for noncovalent aflatoxin:protein adducts in hepatocarcinogenesis.

INTRODUCTION

The AFs\textsuperscript{3} are a family of toxic heterocyclic compounds elaborated by the widely distributed food spoilage mold \textit{Aspergillus flavus}. These toxins have been detected as contaminants in a number of agricultural commodities, including peanuts and various grains, especially in areas where climatic conditions and food storage practices encourage fungal growth. The most potent AF, AFB\textsubscript{1}, has been shown to be hepatocarcinogenic in a number of animal species (19, 38, 55, 57) and has been associated with human liver cancer in several areas of the world (40-42, 48, 49).

Factors known to influence susceptibility to AF-induced hepatocarcinoma include sex difference and level of dietary protein. Male rats, when concurrently subjected to the same dosing protocol as females, are more sensitive to the carcinogenic action of AF (11, 56). Similarly, epidemiological studies implicating AF as a human hepatocarcinogen have indicated a greater propensity toward tumor development in males (40-42, 48, 49). The influence of dietary protein level on AF-induced hepatocarcinoma in the rat was first reported by Madhavan and Gopalan (34) who, comparing 2 levels of dietary protein, obtained from Amersham Corp., Arlington Heights, Ill., and Liquiscint scintillation cocktail was from National Diagnostics, Somerville, N. J. The labeled AF, which was shipped as an ethanolic solution, was evaporated to dryness under N\textsubscript{2} before use. The purity of the AFB, was ascertained using thin-layer chromatography with chloroform:acetone, 9:1, as the developing solvent. Prekotes thin-layer chromatography plates, coated with 250 \(\mu\)m of Adsorbosil 5, were procured for this purpose from Applied Science Laboratories, Inc., State College, Pa. NCS tissue solubilizer was obtained from Amersham Corp., Arlington Heights, Ill., and Liquiscint scintillation cocktail was from National Diagnostics, Somerville, N. J. Spectra/Por 3 membrane tubing was supplied by Spectrum Medical Industries, Inc., Los Angeles, Calif. Disodium EDTA, HEPES, bovine serum albumin, calf thymus DNA, and calf thymus histones were purchased from Sigma Chemical Co., St. Louis, Mo. Acrylamide, bis-acrylamide \([N,N'\text{-methylene}(acrylamide)]\), Coomassie brilliant blue, bromophenol blue, \(N,N',N''\text{-tetramethylethylenediamine}\), and ammonium persulfate were obtained from Bio-Rad Laboratories, Richmond, Calif. All other chemicals used in this study were analytical or reagent grade.

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3 The abbreviations used are: AF, aflatoxin; AFB\textsubscript{1}, aflatoxin B\textsubscript{1}; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HKM, 0.05 M HEPES (pH 7.5), 0.025 M KCl, and 0.005 M MgCl\textsubscript{2}. Several other chemicals used in this study were analytical or reagent grade.
20% casein were prepared by BioServe, Inc., Frenchtown, N. J. The lower protein diet was made isocaloric to the higher by the substitution of sucrose for casein.

Animals. Upon receipt from Charles River Breeding Laboratories, Inc., Wilmington, Mass., weaning male and female F344 rats were housed singly in suspended wire cages and fed the 20% protein diet and water ad libitum. After 1 week, one-half of the animals of each sex were randomly reassigned to the 5% protein diet. Six weeks later, animals were given a 1-mg/kg i.p. dose of [3H]AFB, in dimethyl formamide (5.7 μg AFB, per ml; 0.13 μCi/μg AFB). Two hr following injection, animals were killed by decapitation and their livers were removed.

Preparation of Nuclei. Liver nuclei were isolated by a modification of the method of Blobel and Potter (6). This procedure, and the subsequent isolation and fractionation of chromatin, were carried out at 0–4°C. All buffers used in these procedures contained 0.1 mm phenylmethylsulfonyl fluoride, added just prior to use from a 10 mm ethanolic stock solution. Up to 5 g of liver were minced in 0.25 M sucrose in HKM and homogenized in a Teflon-glass homogenizer (6 strokes at 1700 rpm). Twelve ml of this homogenate were added to a centrifuge tube containing 24 ml of 2.3 M sucrose in HKM. After thoroughly mixing by inversion, the suspension was underlaid by 2 ml of 2.3 M sucrose in HKM and centrifuged in a Beckman SW 27 rotor at 12,000 x g for 1 hr. Two to three strokes at 1700 rpm were used throughout this procedure. After thawing, each chromatin sample was repelletted as above, and the pellet was resuspended in 2 ml of 5% perchloric acid. Following centrifugation in a Sorvall SS-34 rotor at 5000 x g for 10 min, the pelleted chromatin was resuspended, centrifuged, and resuspended again, as above, and frozen at –20°F until fractionated.

Isolation of Chromatin. Chromatin was isolated according to a method described by Chiu et al. (16). Each nuclear pellet was homogenized in 15 ml of NaCl:EDTA [0.008 M NaCl:0.02 M EDTA (pH 6.3)], using a Teflon-glass homogenizer (10 strokes at 1700 rpm). The suspension was centrifuged in a Sorvall SS-34 rotor at 5000 x g for 10 min. The pelleted chromatin was resuspended, centrifuged, and resuspended again, as above, and frozen at –20°F until fractionated.

Fractionation of Chromatin. Chromatin samples were fractionated according to a method detailed by Chiu et al. (16), slightly modified as suggested by Goodwin and Johns (21). Teflon-glass homogenizers (10 strokes at 1700 rpm) were used throughout this procedure. After thawing, each chromatin sample was repelletted as above, and the pellet was resuspended in 2 ml of 5% perchloric acid. Following centrifugation in a Sorvall SM-24 rotor at 20,000 x g for 10 min, the supernatant (S fraction), containing the salt-extractable nonhistones, was removed.

RESULTS

Body Weights and Liver Weights. Upon arrival, male and female rats displayed no significant difference in body weight, as shown in Table 1. Within 1 week, however, the slower growth rate of the females had resulted in an average body weight significantly less than that of the males. During the next 6 weeks, when one-half of the animals of each sex were being fed a 5% protein diet, the expected growth differential between males and females occurred only in those animals fed adequate levels of protein; all animals consuming the 5% protein diet grew at the same depressed rate. At the end of 6 weeks, body weights of males and females fed the low-protein diet were 41

<table>
<thead>
<tr>
<th>Table 1</th>
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<tbody>
<tr>
<td>Body weights and liver weights of male and female F344 rats consuming 5 or 20% protein diets</td>
</tr>
<tr>
<td>Rats, 3 to 4 weeks old, were received from the breeder at the beginning of Week 0. All animals consumed a 20% protein diet until the beginning of Week 1, when one-half of the animals of each sex were randomly reassigned to an isocaloric 5% protein diet.</td>
</tr>
<tr>
<td><strong>Body wt (g)</strong></td>
</tr>
<tr>
<td>Wk 0</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>5% protein</td>
</tr>
<tr>
<td>20% protein</td>
</tr>
<tr>
<td>5.0 ± 0.1</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>5% protein</td>
</tr>
<tr>
<td>20% protein</td>
</tr>
<tr>
<td>6.1 ± 0.1</td>
</tr>
<tr>
<td><strong>No treatment effects.</strong></td>
</tr>
<tr>
<td><strong>Significant treatment effects (p &lt; 0.01) due to sex difference.</strong></td>
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</tbody>
</table>

**Significant treatment effects (p < 0.01) due to dietary protein level and (b) sex difference, with a contributing a greater amount to the observed difference than 5.**

**Mean ± S.E. for 6 animals.**
and 70% of their respective controls. The effects of sex difference and protein level on liver weight followed a similar pattern, while liver weight as a percentage of body weight was altered only by diet, with an increased ratio observed in the protein-deficient rats. AF in Liver. Both dietary protein level and sex difference significantly influenced the amount of AF associated with the liver at the time of sacrifice (Table 2). Within each sex, the low-protein diet decreased liver-associated AF, and within each dietary group, females retained less AF in the liver than did males. These patterns are observed whether the data are expressed as mg AF per g liver or as a percentage of injected dose retained by the liver. Even though animals on the low-protein diet averaged a 14% increase in liver weight as a percentage of body weight (Table 1), they nevertheless averaged a 35% decrease in the percentage of the injected dose associated with the liver.

Protein Composition of Chromatin. For all 4 treatment groups, the percentage of the total chromatin protein contributed by each fraction was determined (Table 3). Neither dietary protein level nor sex difference affected the gross chromatin protein composition as determined by this particular fractionation scheme.

Electrophoretic Patterns of Chromatin Protein Fractions. The electrophoretic pattern of the H fraction (Fig. 1A, Lane 2) is essentially identical to that of purchased calf thymus histones (Fig. 1A, Lane 1). Electrophoresis of the S fraction (Fig. 1A, Lane 3) resulted in 2 bands migrating with an Rf suggesting histone H1. Electrophoresis in adjacent lanes of purchased histone H1 (Fig. 1B, Lane 1), S (Fig. 1B, Lane 2), and purchased histone H1 + S (Fig. 1B, Lane 3) provided evidence that only the H1 component of the S fraction migrates under the conditions described and that this protein comprises approximately 10 to 20% of the total protein in the S fraction. The U fraction (Fig. 1A, Lane 4) contains many proteins having a greater molecular weight than histones. The electrophoretic pattern shown here is very similar to one published by Chiu et al. (17). The proteins comprising the N fraction, like the non-histone components of the S fraction, failed to migrate under the conditions described.

The greatest degree of cross-contamination is within the U fraction (Fig. 1A, Lane 4), in which histones comprise approximately 15% of the total protein.

Association of AF with Chromatin Proteins. In preliminary studies, each of the 4 protein fractions isolated according to this procedure was shown to contain less than 50 μg DNA per mg protein. Thus, the possibility of contamination of the protein fractions with significant quantities of DNA-adducted AF was ruled out.

The association of AF with chromatin proteins was determined both before and after dialysis. The quantity isolated with each protein fraction is referred to as "total AF," while that which remained after dialysis is described as "nondialyzable AF." Preliminary studies demonstrated that treatment of the chromatin protein fractions with dextran-coated charcoal as described by Beato et al. (4) resulted in losses of radioactivity from the various fractions comparable to those obtained by dialysis. Since this charcoal adsorption method is used to

Table 2

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
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<tbody>
<tr>
<td>5% protein</td>
<td>3.0 ± 0.2</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>20% protein</td>
<td>5.4 ± 0.3</td>
<td>4.2 ± 0.1</td>
</tr>
</tbody>
</table>

a Significant treatment effects (p < 0.01) due to (a) dietary protein level and (b) sex difference, with a contributing a greater amount to the observed difference than b. Treatment effects were analyzed by a 2-way analysis of variance.

b Mean ± S.E. for 6 animals.

Table 3

<table>
<thead>
<tr>
<th>Chromatin protein fraction (%)</th>
<th>H</th>
<th>S</th>
<th>U</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% protein</td>
<td>39 ± 0</td>
<td>16 ± 1</td>
<td>42 ± 1</td>
<td>3 ± 0</td>
</tr>
<tr>
<td>20% protein</td>
<td>41 ± 1</td>
<td>16 ± 1</td>
<td>39 ± 1</td>
<td>4 ± 0</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% protein</td>
<td>44 ± 1</td>
<td>13 ± 1</td>
<td>40 ± 0</td>
<td>3 ± 0</td>
</tr>
<tr>
<td>20% protein</td>
<td>41 ± 2</td>
<td>17 ± 1</td>
<td>40 ± 1</td>
<td>2 ± 0</td>
</tr>
</tbody>
</table>

* H, histones, except H1; S, salt-soluble nonhistones, plus H1; U, urea-soluble nonhistones; N, DNA-binding nonhistones.

Mean ± S.E. for 6 animals.

c Mean within each line, means with different superscripts (‘ to *) differ (p < 0.01) according to Tukey's honest significant difference test.
measure the specific binding of steroid hormones to their receptor proteins, the AF:protein bonds not dissociated by dialysis must therefore be, if not all covalent in nature, at least as strong as the interactions binding steroid hormones to their receptor proteins. We cannot be certain that all of the dialyzable AF isolated with each fraction was associated with that fraction in vivo. Although those proteins which remain insoluble during a given extraction retain their aggregated state, it is possible that AF loosely bound to their exposed surfaces could be extracted and thus isolated with the proteins solubilized in that step.

The amounts of both total and nondialyzable AF isolated with the H fraction were consistently less (p < 0.01) than with any other, regardless of sex or level of dietary protein (Table 4). Although this fraction accounted for approximately 41% of the chromatin proteins (Table 3), these proteins bound only 6% of the total AF and 7% of the nondialyzable AF (Table 5).

Both dietary protein level and sex difference significantly influenced the quantities of total and nondialyzable AF associated with each of the protein fractions. Decreased amounts of AF:protein adducts were consistently observed in animals fed a low-protein diet as compared to a high-protein diet and in females as compared to males (Table 4).

In order to determine whether different treatments altered the pattern of AF distribution among the protein fractions, the percentages of the quantities of both total and nondialyzable adducts attributable to each fraction were calculated (Table 5). The percentage of total AF bound to the H and N fractions was independent of both sex and dietary protein level. However, protein-restricted animals bound a smaller percentage of the total AF to the S and a larger percentage to the U proteins than did animals fed 20% protein. There was a similar shift in binding from S to U proteins in females as compared to males. Although a statistically significant effect due to sex difference was observed for nondialyzable binding of AF to the H proteins, an inspection of the data suggests that this effect was very minimal, and it thus appears that the distribution of nondialyzable AF among the chromatin protein fractions remained essentially unaffected by sex difference or protein intake. Therefore, the effects described above for binding of total AF to the S and N fractions must have been due primarily to the dialyzable AF.

The nature of the interactions between AF and chromatin

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Table 4

<table>
<thead>
<tr>
<th>Total AF (pg AF/mg protein)</th>
<th>Nondialyzable AF (pg AF/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H^b,S^b,U^b,N^b</td>
</tr>
<tr>
<td>Male</td>
<td></td>
</tr>
<tr>
<td>5% protein</td>
<td>4.4 ± 0.3^d 50 ± 2^c 49 ± 3^c 28 ± 1^f</td>
</tr>
<tr>
<td>20% protein</td>
<td>8.5 ± 0.6^c 138 ± 6^e 96 ± 4^f 57 ± 2^c</td>
</tr>
<tr>
<td>Female</td>
<td></td>
</tr>
<tr>
<td>5% protein</td>
<td>2.9 ± 0.2^d 23 ± 2^c 33 ± 2^f 14 ± 0^g</td>
</tr>
<tr>
<td>20% protein</td>
<td>5.6 ± 0.1^e 69 ± 3^d 58 ± 2^d 58 ± 3^c</td>
</tr>
</tbody>
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^H, histones, except H1; S, salt-soluble nonhistones, plus H1; U, urea-soluble nonhistones; N, DNA-binding nonhistones.

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Table 5

<table>
<thead>
<tr>
<th>Percentage of distribution of total and nondialyzable AF among chromatin proteins 2 hr following a 1-mg/kg i.p. dose of [3H]AFB, for each treatment, numbers within the lines denote the percentage of distribution of total or nondialyzable AF among the 4 chromatin protein fractions.</th>
</tr>
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<tbody>
<tr>
<td>Total AF (%)</td>
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<tr>
<td>----------------</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>5% protein</td>
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<tr>
<td>20% protein</td>
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<tr>
<td>Female</td>
</tr>
<tr>
<td>5% protein</td>
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<tr>
<td>20% protein</td>
</tr>
</tbody>
</table>

^H, histones, except H1; S, salt-soluble nonhistones, plus H1; U, urea-soluble nonhistones; N, DNA-binding nonhistones.

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proteins varied greatly among different fractions (Table 6). AF binding to proteins in the H and U fractions was much stronger than in the S and N, where most of the protein-associated AF was dialyzable. In those fractions for which treatment effects occurred, the percentage of nondialyzable adducts was greater in protein-restricted animals than in protein-adequate animals and greater in females than in males.

The effects of dietary protein level and sex difference on liver retention of AF (Table 1) and on association of AF with chromatin proteins (Table 4) followed the same patterns. When data from all 4 treatment groups were pooled, liver retention and chromatin protein binding were found to be highly correlated for both total and nondialyzable AF among each protein fraction (Table 7).

**DISCUSSION**

This study has examined the effects of sex difference and dietary protein level on the binding of AF to rat liver chromatin proteins 2 hr following a 1-mg/kg i.p. dose of AFB1, in an attempt to determine whether factors known to affect the susceptibility of rats to AF-induced hepatocarcinoma might alter either gross chromatin protein composition or the patterns of AF adduct formation with these proteins.

The gross protein composition of the chromatin was the same in all 4 treatment groups and did not differ from that determined in preliminary studies using animals not treated with AF. These observations are consistent with the report by Groopman et al. (24) that the electrophoretic profiles of chromatin proteins from control rats and those receiving a 1-mg/kg i.p. dose of AFB1, displayed no major qualitative differences.

The binding of AF to chromatin proteins varied from one fraction to another and was influenced by dietary protein level and, to a lesser extent, by the sex of the animal. In all animals, regardless of diet or sex, AF binding to nonhistones was greater than to histones, both before and after dialysis. This is in contrast to a study by Groopman et al. (24) implicating histones as the major protein targets for nuclear AF binding. Similar studies involving other carcinogens have also resulted in conflicting reports. While Jungmann and Schweppe (29) observed preferential binding of 7,12-dimethylbenz(a)anthracene, N-2-fluorenylacetamide, and N-hydroxy-N-2-fluorenylacetamide to histones, other researchers, studying the binding to chromatin proteins of N-2-fluorenylacetamide (23), N-hydroxy-N-2-fluorenylacetamide (32), benzo(a)pyrene (7, 43), and dimethylnitrosamine (2, 47), all reported greater carcinogen binding to nonhistones. These discrepancies might be due to the use of different procedures for the isolation and fractionation of chromatin proteins. Current knowledge regarding the mechanisms of oncogenesis and the possible involvement of chromatin proteins in this process is too limited to know which protein-carcinogen interactions, if any, might be associated with neoplastic transformation. However, since nonhistones are thought to be much more important than histones in the regulation of differential gene expression (22, 28, 39), it is probable that any specific target proteins of importance to tumorigenesis would be found among the nonhistones.

As noted above, previous studies have demonstrated that females are less sensitive to the carcinogenic action of AF than are males and, similarly, that rats fed a diet containing 5% casein are less at risk than those fed 20% casein. In the present study, sex difference and protein intake altered several parameters of AF distribution in a manner consistent with their effect on tumor incidence. Both the amount of AF per g of liver and the percentage of the dose present in the liver were greater in males than in females and greater in animals fed 20% casein than in those fed 5%. Similarly, the higher-risk factors were associated with greater binding of both nondialyzable and dialyzable AF to the various protein fractions. That the extent of AF binding was strongly correlated with overall carcinogen content of the liver suggests that major effects of animal sex and dietary protein intake occurred at the level of such extra-nuclear events as absorption, metabolism, excretion, and formation of conjugates and/or non-target site adducts. Previous studies investigating the in vitro metabolism of AFB1, by rat liver microsomes have shown that microsomes from male rats form several times more DNA-alkylating metabolites than do those from females (25) and that microsomes from rats fed a 20% casein diet have a greater capacity to metabolize AFB1, than do those from rats fed a 5% casein diet (1). Animal sex and protein
consumption have been shown to similarly affect the extent of adduct formation between AF and various macromolecules. In vitro incubations of AFB1 with calf thymus DNA and liver microsomes from either male or female rats demonstrated greater binding of AF to both the DNA and microsomal protein when the microsomes were from males (26). The in vivo binding of AF to rat liver protein, RNA, and DNA has been shown to be substantially greater in rats maintained on 20% casein diets than in those consuming 5% casein (27). Thus, our finding that the higher-risk factors were associated with greater binding of AF to the various chromatin protein fractions is consistent with previous work.

While the percentage of distribution of nondialyzable AF was not affected by either animal sex or protein intake, dialyzable AF was sensitive to both treatments. The higher-risk animals showed a shift in the distribution of this weakly bound AF from the U protein fraction to the S. Consistent with this was the finding that, in the higher-risk animals, a greater percentage of the total AF associated with the S fraction was dialyzable. This relationship between risk of tumor development and association of dialyzable AF with proteins in the S fraction is a poten-ially significant finding.

Previous investigations from our laboratory (3, 14) have established that the binding of AF to rat liver DNA, RNA, and protein, as well as the amount of AF present in the liver, are linear with respect to dose throughout the range of 1.0 ng/kg to 4.0 mg/kg. Because the degree of adduct formation in these earlier studies was, in the present investigation, highly correlated with the quantity of AF in the liver, it is reasonable to predict that the adduct distribution patterns which we ob-

REFERENCES

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