Effect of Varying Levels of Dietary Protein on Tumor Development and Lipid Metabolism in Rats Exposed to Aflatoxin

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ABSTRACT

Reports in the literature concerning the relationship of protein nutrition to aflatoxicosis are contradictory. In an attempt to elucidate this relationship more clearly, we have examined the effects of low, normal, and high protein-containing diets on tumor incidence and development, as well as on several biochemical indices, in rats which have been exposed to low levels of aflatoxin in a "chronic" rather than "acute" situation. In our study, male weanling rats were place for 3 months on otherwise adequate diets containing either 8, 22, or 30% casein with and without aflatoxin B_1 at 1.7 ppm. Half of the animals in each group received diets which were further supplemented with the amino acid, cystine, at 0.6% of the diet. (Sulfur-containing amino acids are the most limiting amino acids in casein, and the addition of cystine to the diet serves to improve the biological quality of the protein source.) After 3 months the animals were fed control diets without aflatoxin until they were killed at 1 year. Weight gain was markedly decreased and liver weights increased in response to aflatoxin in all groups except those on the low protein diets, where aflatoxin had no effect on these indices. No tumors were found in the livers of rats fed the low protein, aflatoxin-supplemented diet. In the other groups, the severity of the liver involvement increased progressively with increased protein levels in the diet. When cystine was included in the diet, tumors were observed also in the animals fed the low protein diet; furthermore, the livers of those animals on "normal" and high protein diets were much more severely involved than were the livers of animals on non-cystine supplemented diets. Plasma cholesterol levels were increased in response to aflatoxin when the diets containing 22 and 30% protein were fed and when cystine was included in the 8% protein diet. Liver cholesterol levels were increased in response to aflatoxin in all groups except in those receiving the low protein diets. Among these latter animals, aflatoxin administration had no effect on liver cholesterol values. Changes as a result of aflatoxin administration were also observed in the fatty acid composition of sterol esters, triglycerides, and phospholipids of liver and tumor tissue.

INTRODUCTION

For a number of years, the toxicity of aflatoxin to animals, fish, and fowl has been recognized as a serious health and economic problem. At present, it is not known whether man is susceptible to aflatoxin-induced tumors, although there have been several epidemiological studies which suggest that man is not immune (1-3).

Since protein deficiency disease is common in many developing nations and since the available protein source in these areas is usually vegetable protein which may be susceptible to aflatoxin contamination, it becomes of interest to determine whether the level, and the type, of protein in the diet affects susceptibility to aflatoxin toxicity. The existing evidence is controversial. Aflatoxin has been shown to cause a marked inhibition of protein synthesis (4,5). It has also been reported that in short-term experiments, susceptibility to rather large doses of aflatoxin was increased when dietary protein was low (6,7). On the other hand, it has been reported that monkeys receiving small daily doses of aflatoxin for extended periods of time were more resistant to aflatoxicosis when they were consuming low protein diets (8). In addition, studies conducted in our laboratory have suggested that components of the dietary protein source may act, or interact, with other dietary components to modify the severity of the response to aflatoxin (9-11).

In an attempt to possibly resolve this controversy, we undertook an investigation in which diets containing low, "normal," and high levels of protein, with and without aflatoxin, were fed to rats.

EXPERIMENTAL PROCEDURES

The plan of the experiment and the diet used are shown in Tables I and II. Male weanling rats of the former USC strain were fed purified aflatoxin B_1 (purchased from Calbiochem, La Jolla, CA) at 1.7 ppm for 3 months and were thereafter fed a control diet without aflatoxin until sacrificed at 1 year of age. Aflatoxin dissolved in chloroform was incorporated into the diet and the solvent subsequently evaporated. Protein, as casein, was incorporated at either 8, 22, or 30% of the diets and was added to the diets at the expense of sucrose. Since 22% protein is the usual amount in our rat diets, the animals in this group serve as controls for the groups receiving 8% and 30% protein diets.

Cystine was also added to some of the diets at the 0.6% level since sulfur-containing amino acids are the most limiting amino acids of casein. It has been reported by others that animals on low protein diets, where casein is the protein source, develop an acute hepatic necrosis which is the result of cystine deficiency (12). Since it has been shown that a damaged liver is more susceptible to aflatoxin-induced tumors (13,14), the question arose as to whether improvement of the casein, by the addition of cystine, might subsequently alter the resistance of the rats to aflatoxin toxicity. No "acute hepatic necrosis" was observed in the rats fed the low casein diets, although the fact that the addition of cystine to the diets resulted in improved growth

TABLE I

Plan of the Experiment

Group		Protein (%)	Aflatoxin (ppm)	Cystine (%)
8	(6)[a]	8	0	0
8+A	(8)	8	1.7	0
8+CY	(6)	8	0	0.6
8+A+CY	(8)	8	1.7	0.6
22	(6)	22	0	0
22+A	(6)	22	1.7	0
22+CY	(6)	22	0	0.6
22+A+CY	(6)	22	1.7	0.6
30	(6)	30	0	0
30+A	(6)	30	1.7	0
30+CY	(6)	30	0	0.6
30+A+CY	(6)	30	1.7	0.6

[a]Numbers in parentheses are numbers of animals in each group.

TABLE II

Basal Diets

Nutrient	8% Protein	22% Protein	30% Protein
Sucrose	66.75	52.75	44.75
Casein	8.00	22.00	30.00
Salt mix[a]	4.00	4.00	4.00
Solka floc[b]	4.00	4.00	4.00
Vitamin mix[c]	2.00	2.00	2.00
Choline Cl	0.24	0.24	0.24
Corn oil	15.00	15.00	15.00

[a]Wesson Modification of the Osborne-Mendel Salt Mix (22).

[b]Nonnutritive fiber.

[c]The vitamin mix supplied the following mg vitamin/100 g diet: p-amino benzoic acid, 50 mg; inositol, 50 mg; dl, alpha-tocopherol acetate, 10 mg; ascorbic acid, 100 mg; thiamin, 5 mg; calcium pantothenate, 8 mg; niacin, 10 mg; B_{12} (triturate), 6 mg; riboflavin, 3 mg; pyridoxine, 3 mg; 2500 IU Vitamin A; 250 IU Vitamin D_2; folic acid, 1 mg; menadione, 0.5 mg; biotin, 0.2 mg.

and increased organ weights indicates that there might have been a marginal cystine deficiency.

Records were kept of growth, morbidity, and mortality. At the time of sacrifice, gross, as well as histopathological, examinations of the tissues were conducted and organ weights were compared. The following biochemical determinations were carried out using methods reported previously (15,16): plasma and liver cholesterol; total liver lipids; thin layer chromatographic separations of plasma, liver, and tumor lipid fractions; and gas liquid chromatography of fatty acids. The data were analyzed using two-way analysis of variance. Sheffée's method and Student's *t*-test were used to establish 95% confidence limits.

RESULTS AND DISCUSSION

Weight gain during the experiment is shown in Table III. Although rats on the low protein diet gained less weight than those on higher protein diets, statistically significant inhibition of growth as a result of aflatoxin administration occurred only in those animals receiving "normal" protein diets containing added cystine and high protein diets without cystine. At the time of sacrifice, liver, kidney, spleen, adrenals, testes, and heart were removed and weighed. Only the weight of liver was affected by aflatoxin administration (Table III), and this occurred in the cystine-supplemented groups fed the 8 and 22% protein and with and without cystine in the 30% protein group. The observed increases in the weight of the liver in the aflatoxin-supplemented groups were, for the most part, a direct reflection of the severity of the tumor formation and were most pronounced among those rats consuming the aflatoxin-containing 30% casein diet and the 22% casein diet supplemented with cystine.

Table IV shows a summary of the observed pathology. In this table the description "fatty nodules" includes livers with variations and abnormalities in color and texture as well as those containing 1 or 2 large nodular masses affecting one lobe with the remainder of the liver grossly uninvolved, or the presence of numerous small fatty nodules occurring in all lobes but with considerable grossly uninvolved tissue surrounding the nodules. Livers which contained "complex tumors" were characterized by large, multiple tumors and cystic areas in all lobes. These livers were usually 2-4 times normal size and varied in appearance. The tumor masses contained either gray or white fatty material; some contained a network of blood vessels, with and without hemorrhagic areas; some contained cystic areas filled with either yellow or green fluid and some contained hard masses with areas of frank necrosis. In these livers, grossly uninvolved tissue was nearly nonexistent.

Among rats fed the 8% casein diet, there were no liver tumors except in the group receiving the cystine supplement, and here the incidence of tumors was less extensive as compared with other groups. In the groups fed 22 and

TABLE III

Growth and Liver Weights (g)

	8% Protein		22% Protein		30% Protein	
Additive	Wt gain	Liver wt	Wt gain	Liver wt	Wt gain	Liver wt
Control	393 ± 80[a]	11.4 ± 1.5	446 ± 52	13.0 ± 1.0	438 ± 56[D]	12.7 ± 1.8[E]
+A	334 ± 52	10.6 ± 1.3	388 ± 64	22.2 ± 12.3	358 ± 48[D]	41.4 ± 26.7[E]
+CY	362 ± 38	12.0 ± 1.0[A][b]	488 ± 56[B]	14.8 ± 1.4[C]	424 ± 90	12.8 ± 1.8[F]
+A+CY	373 ± 32	16.3 ± 3.1[A]	361 ± 37[B]	50.2 ± 10.5[C]	345 ± 58	45.1 ± 18.2[F]

[a]Standard deviation.

[b]Matched superscripts indicate significant differences between individual groups at $P<0.05$.

TABLE IV

Mortality during Experiment and Occurrence of Pathology in Liver and Kidney of Rats Sacrificed at 1 Year

		Liver tumors			
Group	Deaths	None	Fatty nodules	Complex tumors	Kidney tumors
8	1	5/5[a]	0/5	0/5	0/5
8+A	0	8/8	0/8	0/8	0/8
8+CY	0	6/6	0/6	0/6	0/6
8+A+CY	0	3/8	5/8	0/8	2/8
22	1	5/5	0/5	0/5	0/5
22+A	3	0/3	2/3	1/3	0/3
22+CY	1	5/5	0/5	0/5	0/5
22+A+CY	0	0/6	1/6	5/6	2/6
30	0	6/6	0/6	0/6	0/6
30+A	1	0/5	2/5	3/5	0/5
30+CY	2	4/4	0/4	0/4	0/4
30+A+CY	1	0/5	0/5	5/5	0/5

[a]Number of animals with tumors/number of animals in group.

TABLE V

Cholesterol of Plasma (mg/dl) and Liver (mg/g)

Additive	8% Protein		22% Protein		30% Protein	
	Plasma	Liver	Plasma	Liver	Plasma	Liver
Control	91.3 ± 10.9[a]	2.06 ± 0.30^{F}	90.8 ± 5.6	2.36 ± 0.43	97.1 ± 13.2	3.01 ± 0.63^{F}
+A	79.5 ± 11.9^{AB}	2.20 ± 0.45^{GH}	105.3 ± 18.3^{A}	4.20 ± 2.00^{G}	139.5 ± 55.0^{B}	3.58 ± 0.71^{H}
+CY	90.5 ± 5.1^{CD}	2.52 ± 0.52	108.6 ± 6.5^{DI}	2.00 ± 0.32^{J}	104.0 ± 23.0	2.43 ± 0.26
+A+CY	104.2 ± 9.2^{CE}	2.74 ± 0.73	132.9 ± 14.0^{EI}	3.00 ± 0.30^{J}	123.0 ± 24.0	3.89 ± 0.93

[a]Standard deviation.

[b]Matched superscripts indicate significant differences between individual groups at $P<0.05$.

TABLE VI

Some Major Fatty Acids of the Sterol Ester Fraction of Liver

Group	Fatty acids (%)			
	16:0	18:1	18:2	20:4
8	26.9 ± 2.5^{Aa}	24.3 ± 3.4[b]	22.5 ± 2.8	6.9 ± 0.8
8+A	21.4 ± 3.4^{A}	24.4 ± 4.5	23.5 ± 1.9	8.8 ± 1.7
8+CY	20.4 ± 5.0	26.8 ± 2.3	24.9 ± 3.2	9.7 ± 1.5
8+A+CY	24.5 ± 6.3	24.6 ± 3.0	21.8 ± 4.0	10.1 ± 1.8
22	22.2 ± 7.3	27.9 ± 6.1	19.3 ± 2.2	7.0 ± 1.9
22+A	25.8 ± 4.6	24.1 ± 1.8	19.0 ± 0.6	11.1 ± 3.7
22+CY	31.3 ± 5.7^{B}	20.3 ± 6.9^{E}	15.4 ± 1.9^{G}	11.5 ± 2.5
22+A+CY	14.6 ± 2.6^{B}	32.6 ± 7.2^{E}	24.8 ± 4.2^{G}	8.2 ± 4.1
30	25.5 ± 7.6^{C}	28.4 ± 4.6	18.8 ± 3.4^{H}	6.2 ± 2.3^{I}
30+A	14.4 ± 3.4^{C}	32.7 ± 7.5	28.6 ± 5.0^{H}	10.6 ± 1.5^{I}
30+CY	26.1 ± 4.3^{D}	27.9 ± 1.9^{F}	21.2 ± 7.4	8.3 ± 3.3
30+A+CY	16.8 ± 2.9^{D}	32.1 ± 4.2^{F}	24.4 ± 1.8	7.2 ± 2.5

[a]Matched superscripts indicate significant differences between individual groups at $P<0.05$.

[b]Standard deviation.

30% casein, all aflatoxin-dosed rats had liver tumors, and the severity of the tumors was greatest when aflatoxin was fed with diets containing added cystine. In general, the liver tumors themselves were characterized as being either of complex architecture or composed of compact parenchymatous cells. Histopathological examination of tissue slices from liver, kidney, spleen, adrenals, stomach, testes, heart, and aorta revealed that, with the exception of liver and kidney, aflatoxin was without apparent effect on the various organs.

Liver tumors found in the aflatoxin-treated rats fed the low protein diet supplemented with cystine (8+A+Cy) were well circumscribed and contained large pleomorphic cells. There was no evidence of malignant change. The kidney tumors observed in this group were described as papillary adenoma.

In contrast, aflatoxin-dosed rats fed the 22 and the 30% cystine-supplemented casein diets often showed signs of early cirrhosis of the liver, and it was also fairly common to find foci of necrosis in the liver tumors of these animals. The kidney tumors observed in the group fed 22% casein were nonmalignant tumors of the cortex with necrotic foci near the center of the tumors.

Whereas the incidence and size of the tumors, under these experimental conditions, appears to depend upon diet, the histological architecture of the tumors themselves does not.

Table V contains plasma and liver cholesterol data. In rats fed either normal or high levels of protein, aflatoxin administration resulted in increased levels of cholesterol in both plasma and nontumorous liver tissue, suggesting either increased cholesterol biosynthesis or decreased cholesterol transport or catabolism. In general, levels of cholesterol in both plasma and liver of rats fed the 8% casein diet were lower than those in rats fed 22 or 30% casein diets. In addition, rats fed the low protein diets did not respond to aflatoxin administration with as large increases in cholesterol levels as did those rats on the higher protein diets. Total lipid determinations showed no significant differences between any of the groups.

Liver sterol esters (Table VI) showed significant decreases in palmitic (C16:0) and increases in oleic (C18:1) and linoleic (C18:2) acids among rats fed the 30% casein diet which had been supplemented with aflatoxin. No such changes were observed in the low protein, aflatoxin-supplemented rats. In the rats fed the 22% casein diet, significant changes, similar to those seen when the diet contained 30% protein, were observed only when the diet was also supplemented with cystine. There were no significant changes in either the triglyceride or phospholipid fractions of liver which could be related to aflatoxin administration, and these are therefore not shown.

A comparison of the fatty acids of liver tumors with the fatty acids of the surrounding uninvolved liver showed that in the triglycerides of tumor tissue (Table VII) there is less linoleic acid and more arachidonic acid, as compared with the surrounding liver tissue, in the animals fed 22 and 30% protein, suggesting either a higher rate of conversion of linoleic to arachidonic acid in tumor as compared to liver, or an inability of arachidonic acid to be further metabolized. When phospholipids of tumor were compared with those of liver, there was a trend towards increased levels of monoenes (C16:1 and C18:1) as well as a lower arachidonic acid content in all aflatoxin-supplemented groups except for the 30% protein and cystine-fed animals.

In general, when diets were fed in which the level of protein was the only variable and small amounts of aflatoxin were administered for extended periods of time, it was found that low levels of dietary protein were associated with a marked decrease in both the incidence and severity of tumors. These findings pose some interesting questions.

Several biochemical changes occurred in response to prior aflatoxin administration in the rats fed normal and high levels of protein which were not observed in the rats

TABLE VII

Some Major Fatty Acids of the Triglyceride and Phospholipid Fractions of Tumor as Compared to Liver

Group		Triglycerides		Phospholipids		
		Percent of fatty acids				
		18:2	20:4	16:1	18:1	20:4
8+A	Liver	37.1 ± 4.8[a]	4.0 ± 0.9	1.4 ± 0.4	11.5 ± 0.6	25.1 ± 2.1
	Tumor[b]					
8+A+CY	Liver	36.2 ± 4.2	2.8 ± 0.9	1.7 ± 0.6	10.5 ± 2.2	26.1 ± 4.8
	Tumor[c]	22.0	2.3	4.5	20.9	24.4
22+A	Liver	33.7 ± 6.6	2.8 ± 1.9	1.3 ± 0.3	10.4 ± 1.8	29.1 ± 2.0
	Tumor[c]	32.9	5.8	2.7	16.2	18.2
22+A+CY	Liver	34.4 ± 3.5[Ad]	4.4 ± 2.3	1.4 ± 0.7[B]	15.9 ± 2.1	21.8 ± 5.3
	Tumor	24.9 ± 2.3[A]	5.6 ± 2.3	2.8 ± 0.6[B]	16.1 ± 2.7	20.6 ± 5.8
30+A	Liver	31.7 ± 6.2	4.5 ± 2.4	1.2 ± 0.4[C]	14.2 ± 4.6[D]	25.6 ± 3.9
	Tumor	27.5 ± 8.5	7.1 ± 3.7	3.5 ± 1.9[C]	20.5 ± 4.8[D]	20.8 ± 2.7
30+A+CY	Liver	32.8 ± 6.8	4.2 ± 0.8	3.6 ± 2.9	14.6 ± 1.5	22.6 ± 10.4
	Tumor	28.2 ± 5.6	6.8 ± 3.1	1.3 ± 0.2	15.1 ± 3.9	24.2 ± 4.3

[a]Standard deviation.

[b]Since there were no tumors in this group, there are no data.

[c]N = 1.

[d]Matched superscripts indicate significant differences between individual groups at $P<0.05$.

fed the low protein diet, e.g., elevated plasma and liver cholesterol levels and some changes in the liver fatty acids. The corresponding lack of biochemical response to prior aflatoxin exposure in rats fed the low protein diets may possibly be due to a depression of the activities of the enzyme systems involved as a result of protein deficiency. It is well known that many foreign chemicals are converted to biologically less harmful substances by the enzyme systems of the liver, and it has been shown that these enzyme systems are inhibited in protein deficiency (17,18). It has also been proposed that some metabolite of aflatoxin may actually be the toxic agent involved in aflatoxicosis rather than aflatoxin itself (19-21). If the activities of the enzymes involved in the conversion of aflatoxin to a toxic metabolite were decreased as a result of protein deficiency, a decrease in tumorigenesis could be expected. Our data support this concept and indicate a need for continued investigation to define and further characterize this metabolite.

However, biochemical changes observed exclusively in the rats fed low protein diets which were not associated with aflatoxin administration must not be neglected. It is possible that these biochemical differences may be associated, either directly or indirectly, with the increased resistance of these rats to aflatoxin toxicity.

It is obvious that the problem of dietary manipulation in the control of tumorigenesis is a complex one. Whereas a nutritional deficiency may make an animal more susceptible to infection and possibly to tumor development, it is also possible that nutritional sufficiency or overabundance may promote increased activity of cells or biological systems with possible neoplastic potential.

Since in this study it was found that, under certain conditions, a nutritional deficiency (in this case a low protein diet) may actually be protective when an organism is exposed to an environmental toxin, the practice of indiscriminate nutritional supplementation in areas where nutritional deprivation may be coupled with a contaminated food supply should be reexamined.

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