Dietary Protein Level and Aflatoxin B₁–Induced Preneoplastic Hepatic Lesions in the Rat

GEORGE E. DUNAIF* AND T. COLIN CAMPBELL†,*2

*Field of Environmental Toxicology and †Division of Nutritional Sciences, Cornell University, Ithaca, NY 14853

ABSTRACT Previous studies have shown that the development in rats of aflatoxin B₁ (AFB₁)-induced γ-glutamyl transpeptidase—positive (GGT⁺) foci, indicators of early preneoplastic liver lesions, was markedly greater when a 20% casein diet was fed than when a 5% casein diet was fed during the postinitiation period. In the present study, the dose-response relationship between dietary protein level (dose) and emergence of AFB₁-induced GGT⁺ foci (response) in livers of rats was determined. Male Fischer-344 rats fed a 20% casein diet were orally administered AFB₁ at a dose level of 250 µg/(kg·d) (10 doses over 12 d). One week after the last dose, the animals were divided into eight groups and fed isoenergetic diets containing either 4, 6, 8, 10, 12, 15, 20 or 30% dietary casein for the remaining 12 wk of the study. The development of GGT⁺ foci, as measured by number and percent of liver volume occupied, displayed a response with three discrete phases. The lowest dietary protein levels, 4, 6, 8 and 10% casein, were associated with a minimal level of GGT⁺ foci development. Between 10 and 12% dietary casein, the development of GGT⁺ foci sharply increased, up to the 15–30% dietary casein level. The sudden increase in the formation of GGT⁺ foci at 10–12% dietary casein was just above the level of dietary casein (6–8%) required for maximum body weight gain. These results in this animal model suggest that protein intake in excess of that required to sustain maximum growth rate may enhance AFB₁-induced cancer development. J. Nutr. 117: 1298–1302, 1987.

INDEXING KEY WORDS:

• aflatoxin • hepatocarcinogenesis • dietary protein • γ-glutamyl transpeptidase—positive foci • liver cancer

A higher intake of dietary protein has been shown to enhance the development of aflatoxin B₁ (AFB₁)-induced hepatocellular carcinoma in rats (1). Earlier studies from this laboratory showed that diets containing 20% casein enhanced in vivo activation of AFB₁ when compared with diets containing 5% casein (2). However, later studies showed that despite this dietary protein-dependent correlation between AFB₁ activation and AFB₁-induced tumor formation, the more substantial effect of dietary protein intake occurred after AFB₁ administration, i.e., during the postinitiation period (3, 4).

Significant correlations have been reported for the level of protein intake and several human cancers (5, 6), in fact, these correlations approximate those for total fat intake and human cancers (6). A similar dietary protein effect has been observed for diverse experimental animal tumors that are induced by chemical carcinogens of varied chemical and biochemical properties (6). The protein intake exhibiting a correlation with cancer risk was reported to range from that which meets body maintenance requirements (~ 5% dietary protein) to that which approximates a doubling of growth and maintenance requirements (~ 20–25% dietary protein) (6).

In our laboratory, we have studied the influence of high and low dietary protein intakes on the development of AFB₁-induced preneoplastic liver lesions (3, 4). These preneoplastic lesions are focal areas of hepatocellular alteration that can be identified and quantified by staining histological sections of liver for several types of histochemical markers (7), including γ-glutamyl transpeptidase (GGT) activity. These GGT⁺ positive (GGT⁺) foci are believed to be both the amplified clones of initiated cells (8, 9) and the precursors of later developing hepatocellular carcinomas (10, 11). Previously, we reported that feeding diets containing...
5% casein, compared with 20% casein, significantly inhibited the development of GGT+ foci in livers of Fischer-344 rats [3, 4, 12], analogous to the effect of dietary protein on liver tumors [1]. The present study was designed to characterize more precisely the emergence of GGT+ foci in rats fed discrete levels (i.e., low to high) of dietary casein when fed after the completion of the AFB1 dosing period. This study objective is in accord with the recommendation of the National Research Council, which called for studies to evaluate the dose-response relationships between nutrient intakes and carcinogenic responses [13].

**MATERIALS AND METHODS**

**Animals, diets and chemicals.** Male Fischer-344 rats weighing ~ 40 g were obtained from a commercial breeder (Charles River, Wilmington, MA). All animals were housed individually and received diet and water ad libitum throughout the entire study period. Treatment diets (Dyets, Bethlehem, PA) were AIN-76-based formulations [14] in which sucrose was substituted iso-calorically for casein to yield eight levels of casein: 4, 6, 8, 10, 12, 15, 20 or 30%. These dietary casein levels were confirmed by analysis for total nitrogen content (Agway, Ithaca, NY). AFB1 (Calbiochem-Behring, Los Angeles, CA) was dissolved in tricaprylin at a concentration of 250 μg/mL.

The rats were acclimated to the 20% casein diet for 2 wk before dosing. AFB1 in tricaprylin was administered by gastric intubation at a level of 250 μg/(kg-dose) for a total of 10 daily doses, administered Monday through Friday for 2 consecutive weeks. Eight control animals received tricaprylin only.

One week after completion of AFB1 administration, animals were randomly assigned to one of eight treatment groups and fed diets containing either 4, 6, 8, 10, 12, 15, 20 or 30% casein for the remaining 12 wk of the study. The control group without AFB1 was continued for the remainder of the study on the 20% casein diet.

**Histochemical determination of GGT+ foci.** At the time of killing, slices of liver were frozen on dry ice and stored at ~ 70°C. Frozen cryostat sections (10 μm) were fixed in ice-cold acetone for 24 h. Sections were assayed histochemically for GGT according to the method of Rutenburg et al. [15]. Adjacent sections were stained with hematoxylin and eosin. Foci of GGT+ hepatocytes were counted with a light microscope, and foci diameters were determined with an eyepiece micrometer. To estimate the area of each liver section, its image was projected onto a sheet of paper at a constant magnification with a photographic enlarger. The outline was traced and cut out and its weight was then compared with a 1-cm² reference image projected at the same magnification. The percentage of the section area occupied by GGT+ foci was determined by projecting each liver section onto a sheet of paper and tracing its outline and the outline of its foci. The sections were then cut out and weighed. Next, the foci in each section were individually cut out and weighed. Thus, by dividing the foci weight by liver section weight, the percentage of section occupied by foci is obtained. From these data, the number of foci per square centimeter, percentage of section area occupied by foci and foci diameters were determined. The number of foci per cubic centimeter and the percent volume occupied were calculated from focal transsectional data by the method of Campbell et al. [16].

Statistically significant differences were assessed by the nonparametric procedures (one-tailed) of Wilcoxon as published by Ciba-Geigy [17].

**RESULTS**

Final body and liver weights are presented in Table 1. No statistically significant differences were observed between groups with respect to terminal body weights, except for the lower body weights of the animals fed the 4% dietary casein. Therefore, a level of dietary casein between 6 and 8% was adequate to generate the maximum body weight gain. Food intakes were only estimated (all groups were approximately the same), because we have not found such data to be particularly revealing of any particular insight. In extensive previous work with this model, we have found food intake to be somewhat depressed at dietary protein levels below 6%, in agreement with the slightly lower body weight gain observed here.

The influence of dietary casein level on the development of GGT+ foci, as measured by number and size

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<tr>
<th>TABLE 1</th>
<th>Terminal body and liver weights</th>
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<td>Postdosing diets¹</td>
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<td>Control</td>
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<td>20% Casein</td>
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¹Percentages of dietary casein fed throughout final 12 wk of postdosing period. All groups were fed an identical AIN-76 20% casein-based diet until assignment to these respective treatment diets at 1-wk postdosing.

²Means ± SEM. Means not sharing a common superscript letter are significantly different (P<0.05).
of foci per cubic centimeter (Table 2) and by percent of liver volume occupied by foci (Fig. 1), displayed a similar response. The "percent liver volume occupied" is considered to be the best indication of lesion burden because both the number and size of foci are considered in the measurement. When the percent liver volume occupied data are considered, the lowest levels of dietary casein (4, 6, 8 and 10%) were associated with negligible development of foci. In contrast, foci development sharply increased from 10 to 20% dietary casein. A modest decrease was observed at 30% dietary casein, although there was no significant difference between 16, 20 and 30% casein diets. A typical focus is shown in Fig. 2.

**FIGURE 1** Percent of liver volume occupied by GGT\(^+\) foci as a function of dietary protein intake.

### DISCUSSION

The development of GGT\(^+\) foci, as measured by number of foci and percent of liver volume occupied, displayed a dietary protein-dependent effect (Fig. 1). The negligible development of foci at the four lower levels of protein intake may be related to inhibition of cell proliferation (18, 19), which appears to be required for foci development (20, 21).

In contrast, foci development abruptly increased between 10 and 12% dietary casein level and continued upward to the 20% dietary casein level. The development of GGT\(^+\) foci at 10–12% dietary casein occurred at a level just above that shown to maximize body weight gain (6–8%). Thus the increasing GGT\(^+\) foci response was independent of body weight gain, providing evidence that increasing intake of calories required for growth is not associated with the GGT\(^+\) foci response. Instead, the response appeared to be solely related to the dietary protein intake in excess of that required for maximal growth rate. The compensatory decrease in carbohydrate [half sucrose, half starch] intake with increased protein intake was also not likely to be responsible for the enhanced development of GGT\(^+\) foci. Previous work in this laboratory showed that an effect of an increased sucrose intake, when compared with mixed sugars, was minimal (22); even if there were a confounding sucrose effect, it would likely proceed in the opposite direction (higher dietary sucrose, lower foci response), thus diminishing, if anything, the protein effect. All other nutrient intakes, when compared with energy intake, were equivalent between groups because dietary nutrient composition was based on energy.

The sharp increase in the development of GGT\(^+\) foci between 10 and 12% dietary casein was due to an increase in foci number rather than foci size (Table 2). These data suggest that when foci appear, however few they may be, the rate of growth beyond 10% dietary casein increases in a linear manner up to ~20% dietary casein. Thus the events that trigger the first appearance of foci would appear to be different from those which foster their subsequent growth.

The decrease in GGT\(^+\) foci development at the very high level of 30% dietary casein is in accord with previous work in this laboratory (12). Similar inhibition of tumorigenesis by very high levels of dietary protein has also been reported in other animal models (6, 23–25), which may have been due in part to the physiological burden of excessive protein metabolism (26).

The dietary protein enhancement of the GGT\(^+\) foci response in this and previous studies (3, 4, 12) is in accord with the dose-response relationship shown for dietary protein enhancement of AFB\(_1\)-induced liver tumors in rats (1, 27). In another AFB\(_1\) tumor model, trout, when exposed to AFB\(_1\) during their embryo stage and fed for 9 mo on 40, 50, 60 or 70% dietary protein, yielded a 12, 25, 40 and 68% incidence of hepatocellular carcinoma, respectively (28). It may be instructive to note...
that this dose-response relationship, although at a much higher protein level than that for rats, similarly occurs at levels in excess of the crude protein requirement of 40% (29) required for maximum growth rate.

Although direct extrapolation of these results to human cancer is not possible, the strong correlations between protein intake and several human cancers (5) are nonetheless in accord with these experimental animal observations. Moreover the correlations reported for the human studies span an approximate range of intake from the recommended daily allowance (30) to a three-fold multiple of the RDA. Thus, if these very specific animal data on the preneoplastic response in rat livers are equivalent to an effect of protein intake on the development of any human cancers, the criteria used for the development of the RDA should be changed to reflect the effect of excessive protein intake on this disease process.

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LITERATURE CITED

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