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Inhibition of Skin Tumor Promotion by Restriction of Fat and Carbohydrate Calories in SENCAR Mice[1]

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ABSTRACT

The purpose of this research was to compare the influence of calorie restriction by removal of fat with calorie restriction by removal of carbohydrate on the promotion of skin tumorigenesis in mice. Female SENCAR mice were initiated with 7,12-dimethylbenz(*a*)anthracene (10 nmol, single topical treatment) and fed calorie-restricted diets during and following promotion with 12-*O*-tetradecanoylphorbol-13-acetate (2 μg, topically, twice a week for 20 weeks). Control diet (American Institute of Nutrition-based formulation) was compared with a diet in which calories from fat and calories from carbohydrate were similar [balanced high fat (BHF)] in *ad libitum*-fed groups. Restricted animals were fed diets such that 35% of the calories from fat [high carbohydrate, calories restricted from fat (HCR)] or from carbohydrate (high fat, calories restricted from carbohydrate) were restricted, but other intake was equivalent to the BHF group. Results showed an inhibition of papilloma number in both restricted groups and the inhibition was greatest in the HCR mice. Larger papillomas were observed on mice in the control, BHF, and high-fat, calories restricted from carbohydrate diet groups than on mice in the HCR group. The pattern of carcinoma development was similar in the mice in the freely fed control and BHF groups. Restriction of calories from either fat or carbohydrate delayed the rate and reduced the incidence of carcinoma development. Carcinoma incidence did not differ between mice fed the high-fat, calories restricted from carbohydrate and HCR diets.

INTRODUCTION

A considerable amount of evidence, from both human and animal studies, suggests that high-fat, high-calorie diets increase the risk of cancer (1). Studies on the influence of calorie restriction on carcinogenesis have provided firm data in support of a role of calorie intake in cancer, but the role of dietary fat is far from clear (2). A particularly troubling question has been whether the influence of fat is due only to the calorie contribution of dietary fat or to other properties of dietary fat. Investigations on carcinogen-induced mammary carcinogenesis in rats with low-calorie, high-fat diets (3, 4) suggested that calorie contributions were probably more important than fat contributions in the enhancement of breast cancer. The purpose of the studies reported here was to compare the effects of calories from fat with the effects of calories from carbohydrate on promotion of skin cancer in mice.

Early investigations by Tannenbaum (5) suggested interactive effects of dietary fat and calorie intake on mammary carcinogenesis in mice. More recent studies on DMBA[3]-induced mammary cancer found that high-fat diets enhanced mammary carcinogenesis only when fed freely (6). Significant effects of fat were not observed when calorie intake was kept constant through a modest restriction (6). In addition, enhancement of mammary cancer in rats fed high-fat diet was not observed when calories were restricted in an earlier experiment (4). However, the influence of restriction of a low-fat diet was not assessed.

Previous studies in this laboratory with DMBA-initiated, TPA-promoted skin tumors demonstrated enhanced promotion in mice fed high-fat diet (7) and an inhibition of skin tumorigenesis in mice restricted in diet or in calorie intake (8). This model is particularly useful in separating the initiation and promotion events in carcinogenesis. The studies reported here assessed the influence of dietary calorie restriction during tumor promotion with TPA because our earlier investigation did not find an influence of dietary calorie or total diet restriction on skin tumor initiation by DMBA, but we observed a striking inhibition of promotion by TPA in restricted mice.

MATERIALS AND METHODS

Female SENCAR mice were obtained from the National Cancer Institute, Frederick, MD, at 6 weeks of age. They were fed control purified diet from 6 to 10 weeks of age, randomized into the experimental groups shown in Table 1 at 6 weeks of age, and treated with 10 nmol DMBA in 0.2 ml acetone at 9 weeks of age. One week later they were given the purified diets shown in Table 2 and twice weekly treatments with 2 μg TPA were initiated. TPA treatments were conducted for 20 weeks and the assigned diets were fed for the remainder of the experiment. Animals were killed when any evidence of pain was observed or when there was clear visual presence of a carcinoma. Lesions which could not be classified based on visual observation were evaluated and assigned based on histomorphology. All surviving acetone-treated mice were killed between 55 and 62 weeks after the experimental diets were initiated (65–72 weeks of age). All surviving DMBA/TPA-treated mice were killed at 59 weeks after DMBA (68 weeks of age).

Experimental diets were formulated as shown in Table 2. The control diet was based on recommendations of the American Institute of Nutrition (9, 10). This diet was formulated with dextrin and glucose as carbohydrate sources and the antioxidant was eliminated because we made diet fresh every week and stored it at 4°C between feedings. The diet was made up in agar:water (1:20) because of the high corn oil content of the HFR diet. Agar was added to cold water, the mixture was brought to boil, and the agar was dissolved. After cooling to 57°C the mixed dry components were added with continuous stirring. This mixture was cooled to room temperature and stored at 4°C for no longer than 1 week. The BHF diet was formulated with a similar proportion of calories from fat and calories from carbohydrate such that restriction of either of these components would be possible. The control and BHF diets were freely fed. Portions of the diet were collected, weighed, dried, and reweighed at the time of feeding to determine the amount of dry diet provided. Diet remaining at the end of the feeding period (this was only in the *ad libitum*-fed control and BHF groups) was dried and the amount of diet and calories consumed was calculated.

The HFR and HCR diets were formulated such that when fed at 35% of the calorie intake of the BHF group, the restriction was entirely from fat (HCR) or carbohydrate (HFR). These diets were fed daily at 65% of the average calorie consumption of the BHF group. Mice in these groups received the same protein, fiber, vitamin, and mineral intake that the BHF group consumed.

Carcass nitrogen and lipid were determined as previously described (7). In addition, in mice fed experimental diets for 19 weeks, the electrical conductivity was determined prior to killing and carcass analysis using an EM scan model SA-2 small research animal body composition analyzer (Springfield, IL). Standard EM scan procedures were used on ether-anesthetized mice. Individual mice were placed in the chamber on their ventral side 4–6 times

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[3] The abbreviations used are: DMBA, 7,12-dimethylbenz(*a*)anthracene; BHF, balanced high fat (diet); HCR, high carbohydrate, calories restricted from fat (diet); HFR, high fat, calories restricted from carbohydrate (diet); TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ANOVA, analysis of variance.

Table 1 *Experimental design for addressing effects of caloric restriction on two-stage skin carcinogenesis*

Weeks of age					No. of mice
6	9	10	29	68	
C[a] →				→	15
C →		BHF →		→	15
C →		HFR →		→	15
C →		HCR →		→	15
C →	↓ →		V — V — V	→	45
C →	↓ →	BHF	– V — V — V	→	45
C →	↓ →	HFR/	– V — V — V	→	45
C →	↓ →	HCR/	– V — V — V	→	45

[a] C, *ad libitum* control diet; ↓, treatment with DMBA, V—V—V, twice weekly treatment with TPA.

Table 2 *Experimental diets*[a]

Ingredient	*Ad libitum* diets		Restricted diets	
	Control (%)	BHF (%)	HCR[b] (%)	HFR[c] (%)
Corn oil	4.8	18.1	1.8	29.0
Casein	19.1	22.6	26.7	36.2
DL-Methionine	0.3	0.3	0.4	0.5
Glucose	14.3	10.2	12.1	2.0
Dextrin	47.7	34.0	40.4	6.9
Fiber[d]	4.8	5.7	6.7	9.3
American Institute of Nutrition mineral mix	3.3	3.9	4.7	6.4
American Institute of Nutrition vitamin mix	0.9	1.2	1.4	1.9
Choline bitartrate	0.2	0.2	0.3	0.4
Agar	4.6	3.9	5.5	7.4
Total	100.0	100.0	100.0	100.0

[a] Ingredients were obtained from Teklad Test Diets, Madison, WI.
[b] HCR mice received 0.81 g of this diet for every g consumed by the *ad libitum* BHF group.
[c] HFR mice received 0.6 g of this diet for every g consumed by the *ad libitum* BHF group.
[d] Nonnutritive fiber, purified wood cellulose (74–75% crude fiber).

each day for 3 sequential days and the values were averaged. SD ranged from 2.1 to 4.5% on means from an individual mouse.

Data on body weight, food consumption, carcass analysis, and papilloma development were analyzed by ANOVA using the appropriate model (11). Variation is reported as SEM based on the residual mean square from the ANOVA. Comparison of individual values was conducted using a *t* test based on the residual mean square from the ANOVA. Carcinoma development was compared by Kaplan-Meier (12) cumulative incidence analysis using: (*a*) data sets including all mice with the assumption that the mice killed before the termination of the experiment because of causes other than carcinoma had the risk of developing carcinoma of the remaining mice; or (*b*) data sets with the mice killed early eliminated. Log rank values were calculated to compare groups (13). In both approaches the results were the same and values from the first approach are given in "Results."

RESULTS

Body weights of DMBA/TPA-treated mice from each dietary protocol are shown in Fig. 1. Statistical analysis was performed at weeks 20, 40, and 59 after DMBA treatment. Mice on the diet-restricted protocols (HCR and HFR) weighed less than mice on the *ad libitum*-fed protocols at each of the time points analyzed ($P < 0.001$). There were no significant differences in body weight between the mice fed the control and the BHF diet or between mice fed the HCR or the HFR diet. DMBA/TPA treatment reduced body weight in all groups with an overall reduction of 5% ($P < 0.001$).

Calorie consumption of the *ad libitum*-fed mice did not differ between the DMBA/TPA-treated mice and acetone-treated animals or between the control and BHF groups early (weeks 5–10 following DMBA) during promotion with an overall average of 19.9 ± 0.7 kcal/day. Late during promotion (weeks 40–45 following DMBA), however, calorie consumption was highest in the DMBA/TPA-treated BHF group (20.8 ± 0.6 kcal/day), intermediate in the DMBA/TPA-treated control group (18.3 ± 0.6 kcal/day), and lowest in the groups fed control or BHF diet and treated with acetone (17.7 ± 0.7 kcal/day). Food consumption of the restricted mice was at the level of 65% of the consumption of the BHF mice. The restricted mice consistently consumed all of their diet allotment.

Body composition as determined by carcass analysis after 19 weeks on the experimental diets in mice not treated with DMBA or TPA is shown in Table 3. The percentages of body water and fat were not significantly influenced by the diets, but body fat tended to be higher and body water tended to be lower in the mice fed the high-fat diets (BHF and HFR) ($P < 0.10$). Carcass nitrogen was highest in the *ad*

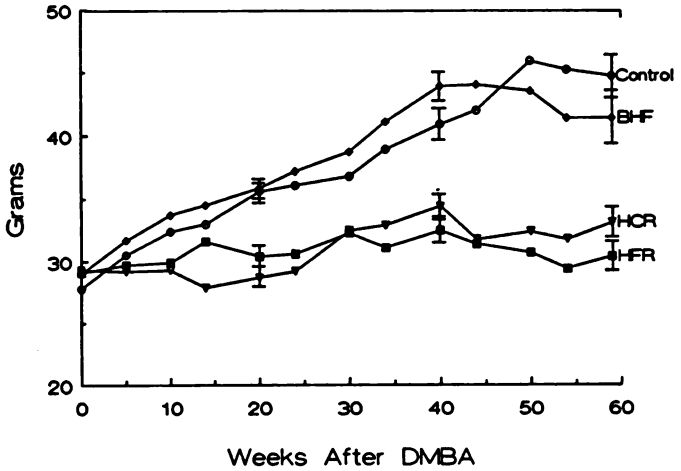

Fig. 1. Body weights of *ad libitum* and restricted mice. Data for mice treated with DMBA and TPA. Values represent the mean ± SEM (*bars*) of 38–42 mice/group at week 0 and 5–16 mice/group at week 60. Control and BHF values were significantly greater than HCR and HFR values at 20, 40, and 60 weeks.

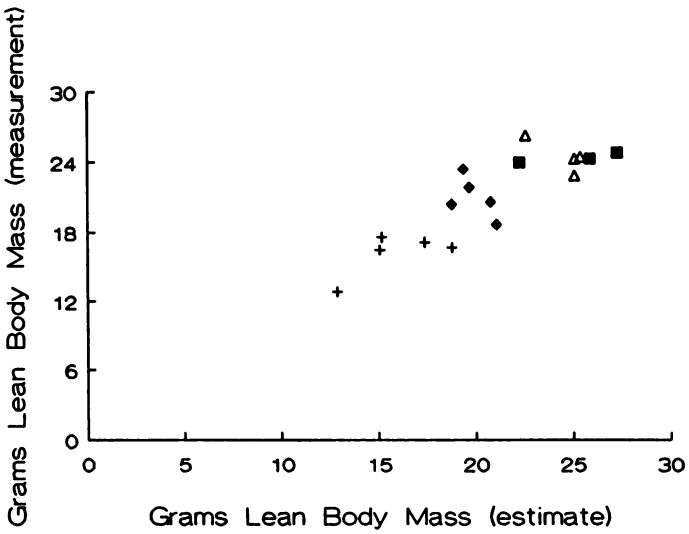

Fig. 2. Relationship between lean body mass as determined by nitrogen content and body water and as determined by electrical conductivity. Data from individual mice fed experimental diets for 19 weeks. △, control; ■, BHF; +, HCR; ◆, HFR. The best fit equation for converting electrical conductivity to g lean body mass (LBM) was developed using the manufacturer's instructions:

$$LBM = 0.418\,(E) - 11.149$$

LBM as calculated by this equation has a SE of 2.2 grams. The correlation coefficient between determined and estimated LBM as $r^2 = 0.87$.

Table 3 *Body composition as determined by carcass analysis of mice fed ad libitum (control and BHF) and diets restricted in calories from fat (HCR) or carbohydrate (HFR)*[a]

Experimental wk	Diet treatment	N	% of body water	% of body fat	g N/100g carcass
19[b]	Control	4	61 ± 3	13 ± 3	2.6 ± 0.2(b,c)
19	BHF	4	57 ± 3	17 ± 3	2.9 ± 0.2(c)
19	HCR	5	63 ± 3	12 ± 3	1.5 ± 0.2(a)
19	HFR	5	58 ± 3	17 ± 3	2.2 ± 0.2(b)
59[c]	Control	5	52 ± 3(d)	24 ± 4(e)	4.6 ± 0.4(e)
59	BHF	6	61 ± 3(e)	16 ± 3(d,e)	4.3 ± 0.4(e)
59	HCR	10	58 ± 2(d,e)	15 ± 3(d,e)	3.1 ± 0.3(d)
59	HFR	10	64 ± 2(e)	10 ± 3(d)	2.3 ± 0.3(d)

[a] Values presented as mean ± SEM; separate statistical analyses were conducted on mice in the two experimental groups (19 and 59 weeks). Letters in parentheses indicate significant differences: a<b<c; d<e; $P < 0.05$. Values with two letters in parentheses are not significantly different from values with either of those superscripts.

[b] Mice were fed the diets from 6 weeks of age until 25 weeks of age. They were not treated with topical agents, a 2- x 2-cm area of skin was removed prior to analysis.

[c] Mice were treated with DMBA + TPA and survived until the end of the tumorigenesis study at 59 weeks after DMBA. These mice did not develop carcinomas.

libitum-fed control and BHF groups and lowest in the HCR group. Measurements of electrical conductivity on these mice showed a correlation ($r^2 = 0.87$) between g of lean body mass (body water plus body protein calculated from g of nitrogen) and as determined by conductivity as shown in Fig. 2. Body fat determination and estimates from EM scan were poorly correlated ($r^2 = 0.7$). Body composition of DMBA/TPA-treated mice as determined by carcass analysis at 59 weeks after DMBA treatment is shown in Table 3. The percentage of body water was higher in mice fed the high-fat diets (BHF and HFR) than in the control mice. Body fat was higher in the control mice than it was in the HFR group but significant differences were not observed between the HCR and HFR mice. Nitrogen content was higher in the *ad libitum*-fed mice than in the restricted groups.

Papillomas appeared at week 6 in the control and BHF group and at weeks 8 and 10 in the HFR and HCR groups, respectively. Papilloma numbers/effective mouse are shown in Fig. 3. Analysis was performed at weeks 14, 20, and 28, and significantly more tumors were induced in the control and BHF group than in the HFR group which had more tumors than the HCR group at each of these times. Papilloma sizes at weeks 10, 14, 20, and 24 are shown in Fig. 4. Sizes were similar at each of the time points in the animals that were fed BHF, HFR, and control diets, but smaller papillomas were observed on the mice fed HCR diet. This is apparent in the larger proportion of small papillomas (<0.1 cm) on the HCR mice and the smaller proportion of large papillomas (≥0.2 cm) at weeks 14, 20, and 24. Papillomas were not observed on acetone-treated mice.

Carcinoma incidence is shown in Fig. 5. Analysis of the cumulative incidence of carcinoma at week 59 by Kaplan-Meier demonstrated significantly fewer carcinomas developing in the HCR and HFR groups than in the control and BHF groups (log rank $P = 0.002$ for analysis including all mice surviving past week 25). Mice were killed because of acetone burns (13% DMBA/TPA-treated mice and 9% of acetone-treated mice) in respective diets before 25 weeks after DMBA was eliminated from this analysis. Papilloma rate on the DMBA/TPA-treated mice eliminated were the same as the rate shown at week 14 in Fig. 3. The BHF and control results were not significantly different.

DISCUSSION

The results of this study indicate a striking inhibition of both papilloma and carcinoma stages of TPA-induced tumor promotion in the skin of mice fed calorie-restricted diet. Inhibition of papilloma and carcinoma rates was observed whether the calories were restricted from carbohydrate or fat. Furthermore, feeding a HFR diet (calories removed from carbohydrate) resulted in more papillomas than did feeding HCR diet (calories removed from fat), although the HFR and HCR diets did not differ in their ability to inhibit carcinoma development. Our data did not suggest differences in progression of papillomas to carcinomas between the dietary groups; however, it is important to note that these studies were not designed to clearly assess dietary effects on progression. Sixty-nine-71% of mice with papillomas developed carcinomas in the control, BHF, and HCR groups, while 48% of the papilloma-bearing HFR mice developed carcinomas (not significantly different).

These results, together with our earlier observations on the influence of high-fat diet (7, 14) or calorie restriction (8) on skin tumor promotion, suggest that the inhibition of skin carcinogenesis by calorie restriction is much more reproducible than the enhancement of cancer in mice fed high-fat diet. This conclusion is evident from comparison of our studies on high-fat effects on mouse skin carcinogenesis. In our first study with freely fed mice (14), we observed larger papillomas on mice fed high-fat diet but the rate of papilloma development was similar between mice fed control and high-fat diet. In a more extensive study on the influence of high-fat and control diet fed at constant calorie intake (7), we observed a clear enhancement of papillomas in mice fed the high-fat diet and an acceleration of carcinoma rates in mice fed the high-fat diet, but the final incidence of carcinoma did not differ between mice fed control and high-fat diet. In the present study with *ad libitum*-fed agar-based diets, we observed no difference in the number of papillomas on mice fed the control or the BHF diet, but BHF mice developed carcinomas slightly earlier [not statistically significant, but similar to the difference observed in our earlier experiment (7)]. With mice fed restricted, agar-based diets, we observed elevated rates and larger sizes of papillomas on mice fed high-fat diet in comparison to mice fed high-carbohydrate diet, but differences in carcinoma development were not observed. Calorie restriction, in contrast, has consistently reduced both papilloma and carcinoma rates and delayed carcinoma development (8).

The inconsistency of the influence of dietary fat effects on skin carcinogenesis is even greater when results of other laboratories are considered. For example, Locniskar *et al.* (15–17) repeatedly observed that diets high in linoleic acid could inhibit skin tumorigenesis. However, they use lower total fat in their diets, keep total fat content constant, and wait for 4 weeks between treatment with DMBA and beginning TPA treatments.

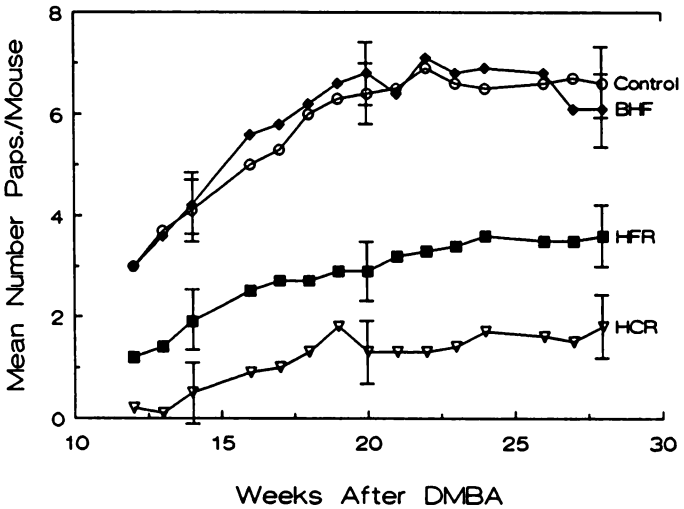

Fig. 3. Papilloma numbers on *ad libitum* and restricted mice. Data for mice treated with DMBA and TPA. Values represent the mean ± SEM (*bars*) of 38–42 mice/group at week 14 and 35–40 mice/group at week 28. Control and BHF values were significantly greater than HFR, and HFR values were greater than HCR values at 14, 20, and 28 weeks ($P < 0.05$).

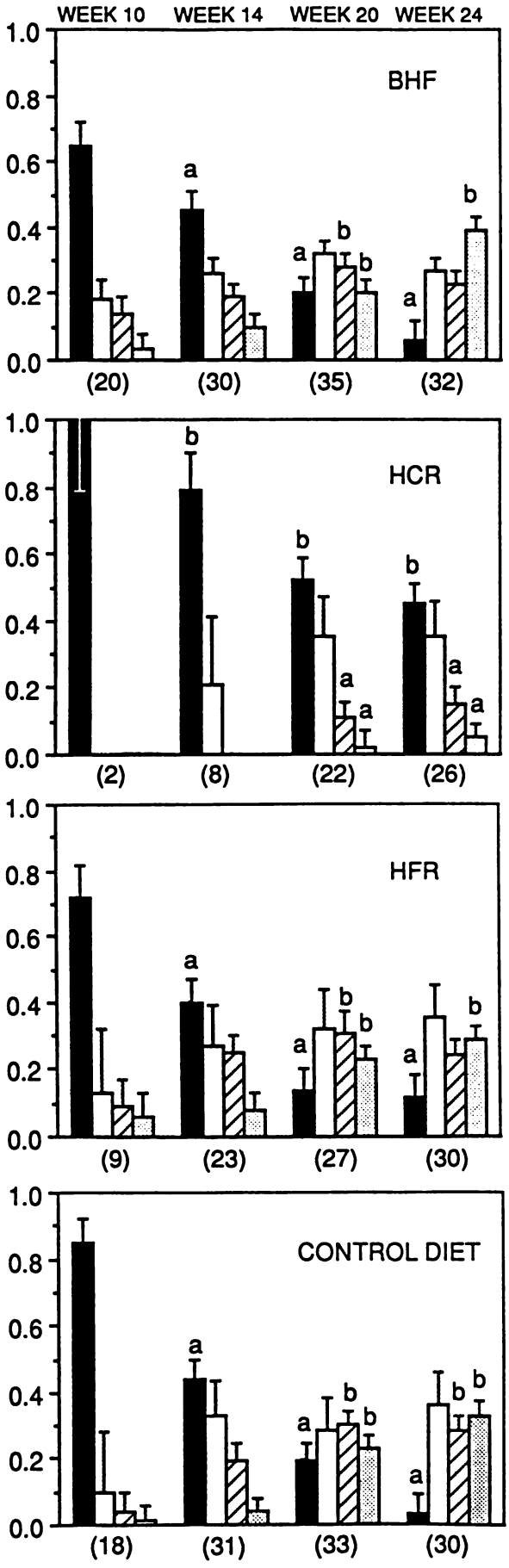

Fig. 4. Papilloma sizes on *ad libitum* and restricted mice. Data are shown as the proportion of papillomas in a given size on mice treated with DMBA and TPA. Values represent the mean ± SEM (*bars*) of the number of mice indicated in *parentheses*. Papilloma sizes: ■, <0.1 cm; □, >0.1<0.2 cm; □, >0.2<0.4 cm; □, 0.4 cm. Values compared between diet groups at a given time interval are significantly different by ANOVA when $a < b$ ($P < 0.05$).

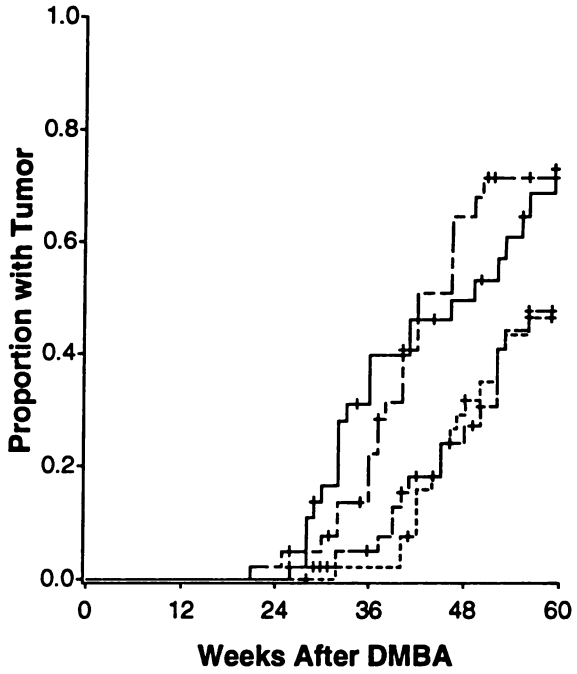

Fig. 5. Time to carcinoma in weeks on *ad libitum* and restricted mice. Data are shown for mice treated with DMBA and TPA. Dietary groups observed for carcinomas consisted of from 23 to 33 mice. Data analysis by Kaplan/Meier estimation of time to carcinoma demonstrated significant delay in the development of carcinomas in the HCR and HFR groups as compared to the control and BHF groups (log rank $P = 0.002$). ——, BHF; – – –, control; - - -, HFR; · · · · ·, HCR.

Results of Welsch *et al.* (6) in studies on mammary carcinogenesis suggest that calorie and fat effects on mammary carcinogenesis are interdependent. Effects of high-fat diet were clearly observed in *ad libitum*-fed rats with a 50% increase in the number of tumors and more than a doubling in the weight of mammary carcinomas induced. Although small elevations were observed in slightly (12%) restricted animals, these differences were not statistically significant. However, clear effects of restriction of calories on mammary carcinogenesis were reported (18) with restrictions of calories from carbohydrate by 20, 30, or 40%. A comparison of restriction of fat with restriction of carbohydrate calories on mammary carcinogenesis has not been reported.

Results from the present experiment did not support a difference in utilization of fat and carbohydrate calories for body weight or for body composition. Mice fed the restricted diets weighed less and had a lower nitrogen (and thus protein) content than freely fed mice. However, there was no consistent or appreciable difference between the mice fed high-fat (HFR)- or high carbohydrate (HCR)-restricted diets or between mice freely fed the control or BHF diets. These data are not in agreement with the observations of Boissonneault *et al.* (4) where rats fed high-fat diets, either restricted or *ad libitum*, contained elevated carcass fat. Our data with mice do not support the suggestion (19) that the relative calorie values for carbohydrate and protein are incorrect. However, our mice were maintained for 59 weeks on the experimental diets and results reported for NZB × NZW F_1 (*B/W*) mice fed diets with carbohydrate or fat calories restricted (20) found

elevated body weight in the mice fed high-fat restricted diet after only about 15 months. The greatest difference was observed after 24 months on the experimental diets (20). In addition, the hypothesis regarding relative differences in metabolically available energy from fat and carbohydrate was based on studies using more rapidly growing rats fed limiting diets (19).

Our results suggested that electrical conductivity measurements could be useful for estimation of lean body mass in mice weighing 20–40 g. Electrical conductivity was compared with carcass analysis only in mice fed experimental diets for 19 weeks. However, calculating body fat by the difference between total body weight and lean body mass as suggested by the vendor was not an acceptable estimate of body fat content.

Kubo *et al.* (20) compared the effect of restriction of fat calories with restriction of carbohydrate calories on longevity and autoimmune-based renal disease in autoimmune-prone NZB × NZW F_1 (*B/W*) mice. Their study revealed a doubling of longevity in mice fed high-fat, 40% calorie-restricted diet, and a 3-fold increase in longevity in mice fed the high-carbohydrate, 40% calorie-restricted diet in comparison with freely fed controls. The onset of high-grade proteinuria as an indication of autoimmune renal disease followed the same pattern. It is notable that there were numerous differences between their restricted diets in addition to the difference in calorie source (20). In a recent report (21), high-fat (45% energy from fat) and low-fat (25% energy from fat) diets were fed freely to Sprague-Dawley rats and at a level of 30% restriction beginning when methylnitrosourea-induced tumors were 1 cm^3 in size. Their results demonstrated a reduction in tumor number and size in the restricted rats and the reduction was greatest when the low-fat diets were fed. These results suggest that autoimmune disease as well as tumorigenesis may be preferentially delayed or inhibited by restriction of carbohydrate or fat calories and that fat calories are somewhat more effective in supporting these disease processes than carbohydrate calories.

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