Calorie Consumption Level Influences Development of C3H/Ou Breast Adenocarcinoma with Indifference to Calorie Source (42984)

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Abstract. **To analyze simultaneously the influence attributable to calorie consumption level and percentage of dietary fat on the spontaneous development of mammary adenocarcinoma, virgin female C3H/Ou mice were separated into five dietary groups. Four groups of mice were fed purified diets either *ad libitum* (16–18 kcal/mouse/day) or restricted 40% in calorie consumption (10–11 kcal/mouse/day), and diets contained either 4.5%, 7.5%, 67%, or 68% calories from fat. Mice that consumed isocaloric diets developed breast malignancy at a comparable pace. Consuming a diet in which fats were present only at levels sufficient to satisfy the threshold requirement of essential fatty acids, 4.5–7.5% of the total calories, or alternatively where dietary fat represented greater than 67% of the total calories consumed, did not significantly alter the tendency for breast tumor development. The pace and frequency with which tumors occurred reflected the host's level of calorie consumption. Mice consuming a high caloric diet, low or high in fat, tended to have a shortened latency to breast tumor formation, an increased incidence of breast tumors, elevated serum prolactin levels, elevated levels of antibodies to mouse mammary tumor virus, and elevated circulating immune complex levels.**

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The risk modulators and the demographic characteristics of human breast cancer implicate genetic and hormonal factors in its etiogenesis; yet, discrepancies in the rate of breast cancer between nations, among ethnic and religious groups, and changes in its incidence among migrants suggest that external factors, perhaps dietary differences, also contribute to its development (1, 2).

Although the positive correlation between national per capital dietary fat availability and breast cancer incidence is qualified support for an association between the two, such evidence is considered weak (1, 3). Case-control, cohort, and analytic epidemiologic studies provide neither uniform nor strong evidence of a positive relationship between dietary fat and breast tumorigenesis (1, 3, 4).

Experimental attempts to elucidate a causal relationship between dietary fat and the genesis of breast malignancy have shown that diets high in fat enhance the development of 7,12-dimethylbenz(*a*)anthracene or *N*-nitroso-*N*-methylurea-induced mammary malignancies in F344 or Sprague-Dawley rats (5–8). Since it has also been shown recently that calorie restriction following chemical induction markedly suppresses tumor development despite an increased proportion of fat in the restricted diet, the promoting effect of fat may be due principally to its greater caloric density (9–12).

The C3H/Ou mouse is a model strain for investigating the biology of spontaneous mammary tumorigenesis. This strain was separated from the C3H/He subline in 1952 prior to that line's lipopolysaccharide mutation and has recently achieved subline designation. Tumors occur in 50% of virgin female mice by 35 weeks of age and are associated with a milk-transmitted type B retrovirus designated mouse mammary tumor virus (MMTV), which is genetically distinct from endogenous proviral sequences, does not contain an oncogene, but is frequently integrated into the host genome near one or two common integration sites (13).

Precedent nutritional studies using C3H stock mice have often been limited by their use of natural ingredient or semipurified diets, excessive restriction of calories, by their evaluating only the effect of calorie level without considering dietary composition, or by only

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comparing accumulated tumor incidences of different dietary groups at single or multiple end points without regard for the pace of tumor development (14–17).

With these limitations in mind and since the development of tumors requiring chemical induction may be subject to the effects of dosage and may be influenced by nutritional modulations which alter the metabolic activation of the carcinogen (18, 19), the following experiments were conducted.

C3H/Ou mice were used as a model of spontaneous breast tumor development. Purified diets were designed so that they contained equivalent amounts of essential vitamins, minerals, an adequate level of essential fatty acids, and 30% calories as protein. Diets varied only in the level of total calories offered and in the principle source of those calories. Mice were fed purified diets either *ad libitum* (16–18 kcal/day) or restricted 40% in calorie intake (10–11 kcal/day). To evaluate simultaneously the relative contribution of the calorie consumption level and the percentage of dietary fat, mice consuming each of the two calorie intake levels were separated into two groups and given diets either low or high in fat content, i.e., where approximately 60% of the total calories were derived from either carbohydrate (low proportion of fat in the diet) or from fat (high proportion of fat in the diet).

Our findings show that calorie intake level has an overriding influence on the development of C3H/Ou breast adenocarcinoma and that breast tumorigenesis proceeds with relative indifference to calorie source. Total calorie restriction of 40% delays greatly the onset and reduces dramatically the incidence of breast malignancy regardless of the proportion of fat in the calorie-restricted diet. *Ad libitum*-fed C3H/Ou mice developed breast cancer after a short latency whether they were fed diets high in fats, or so low in fat that only the minimal threshold of essential fatty acids was satisfied.

Materials and Methods

Mice. Inbred 6- to 8-week-old virgin female C3H/Ou mice were obtained from Dr. Outzen at the Prehn Laboratory, San Jose, California, and maintained in accordance with the principles described in PHS/NIH publication 86-23.

C3H/Ou mice were separated into four experimental groups of 43 mice fed purified diets. One control group of 36 mice was fed laboratory chow. Mice fed purified diets restricted in calories were individually housed, mice fed purified diets *ad libitum* were housed in pairs, and chow-fed mice were housed in groups of five.

Diets. Four purified diets with compositions as described in Table I were prepared weekly and stored at 4°C until fed.

Isocaloric dietary groups were denoted by capital letters; the *ad libitum* group by the A and the 40% calorie restricted group by B. Subscript 1 denoted a carbohydrate-based diet where approximately 60% of the calories offered were from carbohydrates. Subscript 2 denoted a fat-based diet where approximately 60% of the calories were from fat.

The amounts of essential dietary constituents were calculated with regard to the calorie and gram consumption of foodstuff by mice on the *ad libitum*-fed, carbohydrate-based A_1 diet and by increasing the amount of essentials in the 40% calorie-restricted B_1

Table I. Composition of Diets

Constituent	A_1		B_1		A_2		B_2	
	g	kcal	g	kcal	g	kcal	g	kcal
Sucrose	47.25	189.0	26.51	106.05	—	—	—	—
Glycerol	16.0	64.0	8.98	35.91	—	—	—	—
Lard	—	—	—	—	28.1	252.9	15.77	141.93
Casein	29.4	117.6	17.64	70.56	29.4	117.6	17.64	70.56
Methionine	0.6	2.4	0.36	1.44	0.6	2.4	0.36	1.44
Safflower oil	2.0	18.0	2.0	18.0	2.0	18.0	2.0	18.0
AIN vitamin mix	1.0	3.95	1.0	3.95	1.0	3.95	1.0	3.95
AIN mineral mix	3.5	1.652	3.5	1.652	3.5	1.652	3.5	1.652
Inositol	0.05	0.2	0.05	0.2	0.05	0.2	0.05	0.2
Choline bitartate	0.2	0.8	0.2	0.8	0.2	0.8	0.2	0.8
Total	100.0	397.6	60.24	238.56	64.8	397.5	40.52	238.55
Energy (kcal/g)[a]	3.98		3.96		6.13		5.89	
Protein (kcal/total kcal)[b]	0.302		0.302		0.302		0.302	
Carbohydrate (kcal/total kcal)[a]	0.636		0.595		—		—	
Fat (kcal/total kcal)[d]	0.045		0.075		0.681		0.670	

[a] Based on sucrose, glycerol, casein, methionine, inositol, and choline bitartate at 4 kcal/g and lard and safflower oil at 9 kcal/g.
[b] Based on casein plus methionine calories per total calories.
[c] Based on sucrose plus glycerol calories per total calories.
[d] Based on lard plus safflower oil per total calories.

diet, as well as in the isocaloric, but calorie-dense A_2 fat-based diet and even further increased in the low volume, calorie-dense fat-based B_2 diet. This ensured that even in the small gram amount of foodstuff consumed by the mice in Group B_2, adequate levels of all essentials were consumed.

Equivalent amounts of vitamins, minerals, essential fatty acids, and 30% calories as protein were available in all four purified diets. All constituents were obtained from ICN Biochemicals, Cleveland, Ohio.

Mice were weighed, palpated for tumors, and fed twice weekly. Forty percent calorie restriction was achieved gradually by the sixth week of study when the mice were 12–14 weeks old. The amount of food consumed by *ad libitum*-fed A1 or A2 mice was determined by weighing the remnant foodstuff and subtracting from the volume offered. Leftover food, body weight, and the goal of 40% calorie restriction were considered when determining the volume of food to offer to each of the four dietary groups at the subsequent feeding period.

Mice with tumors were euthanized 3–4 weeks after tumor development. Representative age-matched, non-tumor-bearing mice from each dietary group were euthanized at 41, 46, and 58 weeks of age to assess the effects of these nutritional modulations on specific immunologic parameters, the production of MMTV viral particles, serum prolactin levels, and proviral mRNA expression levels (20).

Cell and Tissue Preparations. Single-cell suspensions of splenic leukocytes were prepared by mincing the spleens in Hanks' balanced salt solution (Gibco Laboratories, Grand Island, NY), passing through gauze, and washing three times.

Collected blood was allowed to clot on ice, clarified, and the serum stored at −70°C until used. Mammary gland, tumor, liver, spleen, skeletal muscle, salivary gland, small intestine, abdominal fat, and heart were snap-frozen in liquid nitrogen for Northern blot analysis (20).

Culture Medium. RPMI-1640 medium (Gibco) with 1 μM sodium pyruvate, 5 mM Hepes, 100 units/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol, and 10% fetal calf serum was employed for cell culture experiments.

Mitogen Stimulation. Mitogen-induced blastogenesis was measured by incorporation of tritiated thymidine into proliferating cells. Preparations used were: 4 μg/ml phytohemagglutinin (Difco), 2.5 μg/ml concanavalin A (Calbiochem), 3 μg/ml Salmonella typhosa lipopolysaccharide (Difco), and 2% v/v pokeweed mitogen (Gibco) in culture medium. All preparations were shown in pilot experiments to produce maximum lymphoid cell responses.

Interleukin 2 Production. Spleen cells (2×10^6) were suspended in 1 ml of RPMI 1640 complete medium supplemented with 2 μg of concanavalin A/ml. The cells were cultured in 24-well tissue culture plates (Linbro) for 36 hr at 37°C in an atmosphere containing 5% CO_2. Cells were removed from the culture supernatants by centrifugation at 1500g for 10 min. Cell-free supernatants were stored at −20°C until the interleukin 2 (IL-2) assay was carried out.

IL-2 Assay. The IL-2 activity of supernatants was determined by quantifying the influence of the supernatants on growth of an IL-2-dependent T cell line (21). HT-2 cells, a BALB/c (H-2^d) sheep RBC-specific IL-2-dependent helper T cell line, were used at 5×10^3 cells/well.

Assay of Circulating Immune Complexes (CIC). To measure serum levels of CIC in mice, the Raji cell radioimmunoassay as adapted for mice was used (23). Results are expressed as μg equivalents of aggregated murine IgG per ml of serum.

Natural Killer Cell Activity. Spleen cells from each group were used as effector cells and YAC-1 cells, a Maloney virus-induced T cell lymphoma originally derived from A/Sn mice, were used as target cells at 100:1 effector to target cell ratio.

Induction of Plaque-Forming Cells. The test for *in vitro* plaque-forming cell response to sheep SRBC was conducted according to the method of Mishell and Dutton (24).

Antibodies to Mouse MMTV. Serum antibodies to MMTV were quantitated using an enzyme-linked immunoabsorbent assay method and reported as an optical density value (25).

Electron Microscopy. Mammary glands and tumor tissues were trimmed and cut into 1.0-mm cubes under a dissecting microscope, fixed in 4.0% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in graded alcohol, embedded in Epon 812, sectioned, and stained with uranyl acetate and lead citrate.

Serum Prolactin Measurements. Serum was assessed for the level of prolactin by double-antibody radioimmunoassay using reagents provided by Dr. A. F. Parlow, Pituitary Hormones and Antisera Center, Harbor-UCLA Medical Center, Torrance, California, following methods described previously (16).

Statistics. The incidences of mammary tumor development were compared by survival analysis using Weibull Distribution (SAS, Proc. Life. Reg.) accounting for all right censored data and by the Kaplan-Meier product-limit analysis of individual groups (26). Mouse body weights and the immunologic parameters of each group were compared by Scheffe's analysis of variance and a two-tailed Student's t test.

Results

Virgin female 6- to 8-week old C3H/Ou mice were separated into one of five dietary groups and fed purified diets as described in Table I or laboratory chow.

Group A_1 mice were fed *ad libitum* a diet in which sucrose served as the principle source of calories (64%

carbohydrate calories) and consumed 16–18 kcal/day. Group B_1 mice were fed 40% fewer calories than A_1 mice, a diet in which sucrose also served as the principle source of calories (60% carbohydrate calories) yet these mice consumed 10–11 kcal/day. In both A_1 and B_1 diets, safflower oil was provided to meet the minimal threshold requirement of essential fatty acids in this very low fat diet where energy from fat represented only 4.5–7.5% of the total kcal. The amount of essential constituents in diets A_1 and B_1 were proportionally similar as was the consumption of protein on the basis of total energy consumed.

Group A_2 mice were fed *ad libitum* a diet in which saturated fat (lard, catalogue no. 902140; ICN Biochemicals) served as the principle source of calories (68% of calories as fat) and consumed 16–18 kcal/day. Group B_2 mice were fed 40% fewer calories than A_2 mice, a diet in which saturated fat also served as the principal source of calories (67% of calories as fat), but these mice consumed 10–11 kcal/day. The amount of essential constituents in diets A_2 and B_2 were proportionally similar, as was the consumption of protein on the basis of total energy consumed.

Although the principle source of calories differed, diets A_1 and A_2 were isocaloric as were diets B_1 and B_2. Equivalent amounts of adequate essentials and 30% calories as protein were provided in all four purified diets.

Changes in dietary allotment were made so that mice consuming the restricted diets grew and gained weight, and so as to ensure that B_1 and B_2 dietary groups consumed 40% fewer calories based on the calorie consumption of the full-fed A_1 and A_2 dietary groups (Table II).

Growth. The average body weights of mice of all five dietary groups were compared at 36 weeks of age, at which time the weights were the most divergent (Fig. 1). Mice fed *ad libitum* the fat-based A_2 diet had a significantly greater average body weight than the weights of mice of any of the other groups, $P < 0.001$. The average body weight of mice fed *ad libitum* the carbohydrate-based A_1 diet was significantly greater

Table II. Changes in Dietary Allotment

	Age (weeks)						
	8	10	14	32	36	44	52
				g/mouse/day			
A_1	4.25	4.25	4.50	4.50	4.50	4.50	4.50
B_1	3.83	3.19	2.70	2.50	2.60	2.80	2.60
A_2	2.75	2.75	2.92	2.92	2.92	2.92	2.92
B_2	2.50	2.07	1.75	1.70	1.75	1.90	1.78
				kcal/mouse/day			
A_1	16.9	16.9	17.9	17.9	17.9	17.9	17.9
B_1	15.2	12.6	10.7	9.9	10.3	11.1	10.4
A_2	16.9	16.9	17.9	17.9	17.9	17.9	17.9
B_2	14.7	12.2	10.3	10.0	10.3	11.1	10.4
				% restriction			
Carbohydrate diets	10	25	40	45	42	38	42
Fat diets	13	28	42	44	42	38	42

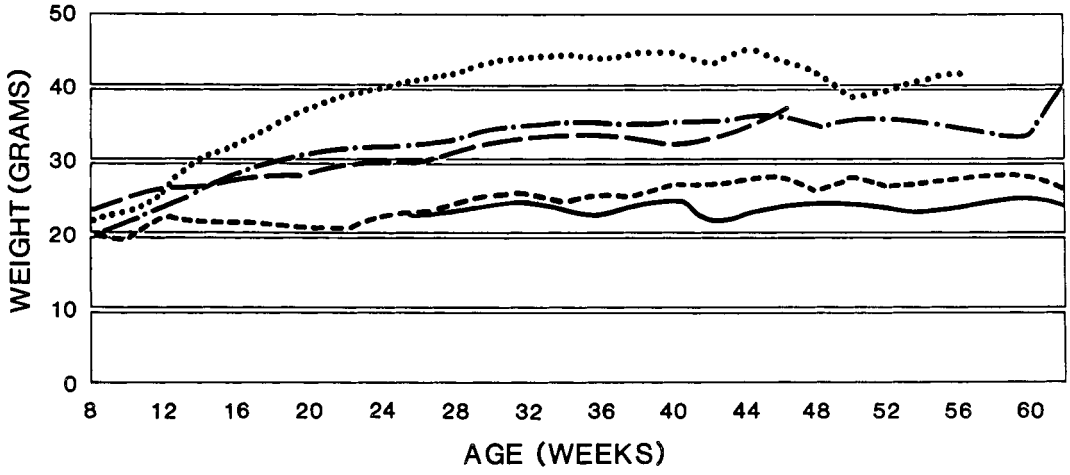

Figure 1. Average body weights of mice fed *ad libitum* the low fat A_1 diet (dot-dash), the high fat A_2 diet (dots), or laboratory chow (long dashes), or restricted 40% in the consumption of calories and fed the low fat B_1 diet (solid line), or the high fat B_2 diet (short dashes).

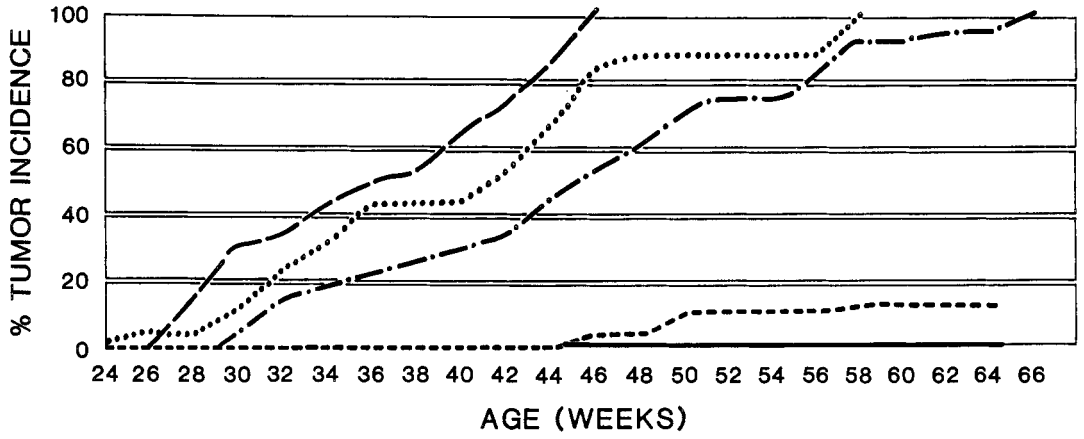

Figure 2. Percentage of occurrence of breast adenocarcinoma among mice fed *ad libitum* the low fat A_1 diet (dot-dash), the high fat A_2 diet (dots), or laboratory chow (long dashes), or restricted 40% in the consumption of calories and fed the low fat B_1 diet (solid line, 0% incidence through 64 weeks), or the high fat B_2 diet (short dashes).

than that of mice fed either the B_1 or B_2 diets ($P < 0.001$).

Incidence of Mammary Adenocarcinoma. As expected for the C3H/Ou strain, the median tumor incidence (MTI) of mice fed laboratory chow was reached by the 35th week of age (Fig. 2) and 100% incidence was reached by the 46th week.

Regardless of whether the percentage of dietary fat was 7.5% (Group B_1) or 67.0% (Group B_2), latency was greatly increased and incidence of mammary tumors markedly reduced among mice restricted 40% in calorie intake (Groups B_1 and B_2), relative to *ad libitum*-fed A_1 or A_2 mice that consumed proportionally comparable diets. Similarly, the rapid and frequent occurrence of breast tumors among *ad libitum*-fed mice occurred whether the percentage of dietary fat was 4.5% (Group A_1) or 68.0% (Group A_2). This abrogation in breast tumorigenesis among the 40% calorie-restricted B_1 or B_2 dietary groups, and the unimpaired progression to a high incidence of breast malignancy between *ad libitum*-fed A_1 and A_2 groups, occurred irrespective of the fat content of the consumed diet and was intimately associated with the level of calorie intake.

During the 84 weeks of study, only five malignancies developed among mice restricted 40% in calorie intake; one each during Weeks 77 ad 81 among mice fed the primarily carbohydrate-based B_1 diet and one during week 46 and two in week 50 among mice fed the fat-based B_2 diet. In contrast, mice fed purified diets *ad libitum* developed malignancies as early as 30 and 26 weeks in the A_1 and A_2 groups, respectively, and achieved median tumor incidence respectively during Weeks 45 and 41 (Fig. 2).

Although fat-based diets appeared to mildly promote an earlier onset of tumorigenesis among mice fed at either level of calorie intake, this slight left shift in the incidence curve was found to be insignificant. When the tendency for tumor development among mice fed isocaloric diets A_1 and A_2 were compared by survival

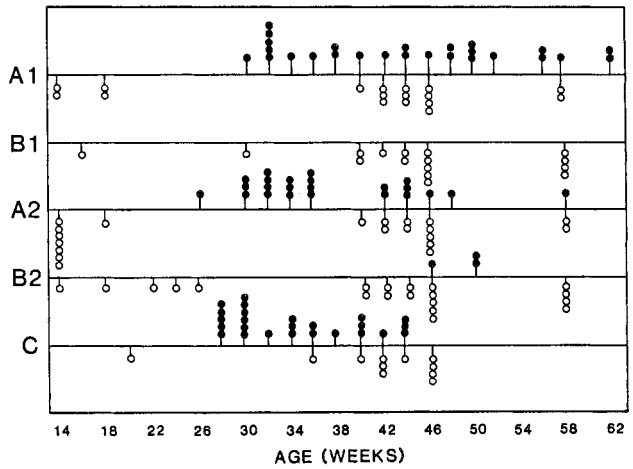

Figure 3. A dot above the time line indicates the occurrence of a breast malignancy in an individual mouse. A dot below the time line indicates euthanasia or death before malignancy (censored from study). At the 66th week of study, 17 of the original 43 mice in Group A_1 had been censored, leaving 26 at risk, of which 100% had developed breast tumors. Similarly, 16 of 43 had been censored from Group B_1, leaving 27 at risk, of which none had developed malignancies, 20 of 43 had been censored from A_2, leaving 23 at risk, of which 100% had developed breast tumors, and 20 of 43 had been censored from B_2, with three of the 23 mice at risk developing breast tumors.

analysis using Weibull Distribution (SAS, Proc. Life Reg.) to account for all animals until the date of their censoring (i.e., euthanasia due to malignancy or for age-matched nontumor-bearing samples or death), the tumor incidences among the A_1 and A_2 groups were found to be similar ($P = 0.1280$), regardless of the diet's fat content.

All mice were accounted for (Fig. 3). After 66 weeks of study, the accumulated tumor incidence for the mice consuming a high calorie intake in Groups A_1 and A_2 was 100%. In marked contrast, after 84 weeks of study, the accumulated tumor incidence for 40% calorie-restricted mice in Groups B_1 and B_2 was only approximately 10%.

Shortened latency and a greatly increased incidence of malignancy occurred among *ad libitum*-fed mice, and delayed development and reduced incidence among calorie-restricted mice occurred regardless of the source of calories or the diet's proportion of fat.

Immunoparameters. C3H mice on lower calorie intake levels grew, gained weight, and have been shown to exhibit normal estrous cycles and normal immunologic capabilities (16, 27, 28). Immunologic functions were found to be well supported and not greatly different in mice of all five dietary groups (Table III).

Mice that consumed the high calorie A_1 had significantly higher circulating immune complex levels than mice fed the 40% calorie-restricted, but proportionally similar B_1 diet. Mice consuming either the A_1 or A_2 diets *ad libitum* had higher anti-MMTV antibody levels ($P < 0.005$). These findings are thought to reflect the greater rate of virus production and tumorigenesis in these high calorie-consuming groups (Table III).

Microscopic Evaluation. Mammary glands and mammary tumors were evaluated in all five dietary groups by light and electron microscopy. All tumors were adenocarcinomas of the A or B histologic type. Although no formal attempt to quantitate MMTV expression was made, our impression confirmed that the expression of intracellular and extracellular virus particles was more easily recognized in local areas of hyperplasia (i.e., minimal alveolar lesions) and in malignancies, and that these proliferative changes in mammary gland histology were directly influenced by the nutritional status of the host (i.e., calorie intake level).

Prolactin. A very limited number of sera (2–4) from mice without malignancies within each dietary group were evaluated at the 41st–46th weeks for the level of circulating prolactin (PRL). Representative whole serum samples were collected from each group simultaneously by decapitation. Mice consuming a high calorie intake diet with either fat or carbohydrate as the principal calorie source had PRL levels higher than those measured in the low calorie intake mice (approximately 62 ng/ml vs 15 ng/ml). This tendency was supported by a separate subsequent study not described in detail here where C3H/Ou mice were fed A_1 or B_1 diets and PRL levels were determined for five nonmalignant mice in each dietary group on a monthly basis. The PRL levels of *ad libitum*-fed mice tended to be higher, and, in some cases, were significantly elevated when compared with those measured in 40% calorie-restricted mice. These preliminary findings have prompted further investigations now in progress of the mechanisms by which calorie intake level modulates hormonal and genetic factors considered vital to C3H/Ou breast tumorigenesis as described in Discussion.

Discussion

Data from epidemiologic and experimental studies have historically alternated between implicating fat or calorie level as crucial to the genesis of breast adenocarcinoma. Although epidemiologic studies have thus far not clearly elucidated clearly a causal relationship, recent experiments using the rat model of chemical carcinogen-induced breast cancer have unambiguously shown that restricting energy intake dramatically reduces tumor yield regardless of total fat intake (9–12, 29).

Herein for the first time, influences attributable to the level of energy or fat consumption were analyzed simultaneously using a model of spontaneous breast cancer. By using purified diets which varied only in the source and level of calories consumed, and an established mouse model of spontaneous mammary malignancy, we have demonstrated that the level of calorie consumption has an overriding influence on the genesis of mammary adenocarcinoma in the C3H/Ou mouse and that this influence is imposed with relative indifference to the level of dietary fat.

Mice that consumed equivalent amounts of total calories developed malignancy at a comparable pace. Feeding a diet in which fats were present only at levels sufficient to satisfy the threshold requirement of essential fatty acids, 4.5–7.5% of the total calories in diets A_1 and B_1, respectively, or alternatively where dietary fat represented greater than 67% of the total calories consumed, diets A_2 and B_2, did not significantly alter the tendency for C3H/Ou mice to develop breast tumors. Instead the pace and frequency with which tu-

Table III. Immunoparameters of nontumor-Bearing C3H/Ou Mice at 41 Weeks of Age

Assay	A_1	B_1	A_2	B_2	Control
Phytohemagglutinin (4 μg)	73,144	63,566	52,128	70,316	88,518
Concanavalin A (2.5 μg)	272,024	292,005	183,861	207,368	236,263
Pokeweed mitogen (2%)	119,879	91,525	96,206	92,881	127,050
Lipopolysaccharide (3 μg)	145,262	106,763	115,689	105,915	117,173
Plaque-forming cells/10^6	77	80	129	27	99
IL-2 (units)	10.1	8.0	9.9	7.6	12.9
Natural killer (% at 100:1)	19	12	18	13	19
MMTV antibodies (optical density)	0.5247	0.3502	0.4812	0.3934	0.4036
CIC (μg/ml)	692	186	287	134	128
n	6	5	5	5	6

mors occurred reflected the host's level of calorie consumption.

Admittedly, the absolute length of the shortest period of latency and of the time to reach MTI were both slightly shortened when mice were fed the fat-based diets. Comparison of accumulated tumor incidences at any one point of time during the 84 weeks of study reveals a higher incidence of tumors among mice fed fat-based diets. This is consistent with an earlier study (15). But accumulated tumor incidences only consider the accumulated population at risk at that moment. When nontumor-bearing mice are euthanized during the experiment as age-matched controls to provide samples for immunologic, virologic, or endocrinologic evaluations, or if they die unexpectedly, the size of the population at risk and the percentage of tumor occurrence is altered. This artificial raising and lowering of the incidence can be misleading. To accurately assess the tendency for diet to modulate tumor development, it is essential that the entire experimental population be included in the evaluation.

This form of assessment is made possible using a survival analysis program. Fluctuations in population size, the pace of accumulating occurrences, as well as total accumulated occurrences are considered in this form of evaluation. A simple comparison of percentage of incidence at a selected end point does not take into account the pace of occurrence, changes in population size, and the real possibility that a separate comparison at a different end point could lead to an entirely different conclusion.

The slightly shortened latency period to first tumor occurrence, the time to MTI, and the slightly higher accumulated tumor incidences at certain time points of comparison for mice fed diets high in fat (A_2 and B_2) were not significantly different when compared with the rates of occurrence and the total accumulated incidences of breast cancer for mice fed low fat diets (A_1 and B_1), $P = 0.1280$.

The level of antibodies to MMTV and the total CIC levels within sera from mice fed high calorie diets were greater than those of mice fed diets 40% restricted in calories. These findings substantiate an earlier report that the level of circulating anti-MMTV antibodies reflected the level of calorie consumption in the C3H strain (27). These results extend those earlier findings by demonstrating that the level of CIC are similarly affected, and that this effect occurs regardless of the proportion or relative level of dietary fat.

Also verified in this report is the earlier observation that MMTV particle production is influenced by calorie intake level (16), and, furthermore, this report shows that this effect occurs irrespective of dietary fat content. The relationship between virus particle production and calorie consumption could be indirect. The moment at which enhanced viral particle production occurs has not been identified and may be dependent on or precede a change in host cell proliferative rate (i.e., a pathologic hyperplastic foci or malignant transformation).

We have also confirmed that the level of circulating PRL in C3H/Ou female mice is directly influenced by the level of calorie consumption (16), and have further shown that this effect is irrespective of the fat content of the diet. That prolactin plays a role in mammary lobuloalveolar development is established. Less understood is its effects on fetal and neonatal development, the regulation of growth and differentiation, and the immune response. For example, it functions as a potent hepatocellular and T lymphocyte co-mitogen which influences lymphocyte subpopulation development patterns and plays a central role in the regeneration of the liver following partial hepatectomy (30, 31).

The evidence that prolactin contributes directly to mitogenesis of human breast epithelium or breast cancer lines is mixed. Serum prolactin levels have been reported to be higher in some breast cancer patients and in daughters of women with breast cancer, yet other reports find no such elevation (32, 33).

Multiple pituitary isografts, which are principally PRL secreting, to weaned adreno-ovariectomized (foster-nursed) MMTV-free (nursed) C3H mice resulted in MTI in 32 weeks, with 100% incidence by 60 weeks, as opposed to 86 weeks to achieve MTI in nongrafted intact controls (34).

By using Northern blot analysis, we have presented evidence elsewhere that the expression of MMTV-related genes is dramatically influenced by the level of calorie intake irrespective of dietary fat (20). These findings have prompted current investigations of the influence of calories on the synthesis and release of PRL, on the regulation of gene expression, and the assessment of these combined hormonal and genetic influences on breast tumorigenesis in the C3H/Ou mouse.

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