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Low-calorie diet prevents the development of mammary tumors in C3H mice and reduces circulating prolactin level, murine mammary tumor virus expression, and proliferation of mammary alveolar cells

(estrus cycle/thyrotropin/growth hormone/types A and B virus particles)

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ABSTRACT **The effect of calorie intake on the development of spontaneous mammary tumors in virgin C3H mice was studied. Only about 10% of the mice fed a low-calorie diet [10 kcal/day (1 kcal = 4.184 kJ)] since weaning developed mammary tumors, compared to about 60% of those mice that were reared on high-calorie diets (16 kcal/day or lab chow ad lib). In order to understand the mechanism by which a low-calorie diet decreases the occurrence of mammary tumors in mice, we compared the sex cycle, the amounts of circulating thyroid-stimulating hormone (thyrotropin), growth hormone, and prolactin, the production of type A and B virus particles in the mammary glands, and the morphology of the mammary glands of mice fed low- and high-calorie diets. The amount of serum prolactin and the synthesis of type A and B particles in mammary tissues of mice fed a low-calorie diet was markedly decreased compared to those of age-matched mice fed high-calorie diets. In addition, in young mice fed a low-calorie diet, there were fewer mammary alveolar lesions than in mice fed a high-calorie diet, although the size of the lesions was similar. However, in older mice fed the high-calorie diet, the number and size of these lesions were greater than in the mice raised on the low-calorie diet. The other factors that we studied were not affected by calorie restriction. Our findings suggest that the reduction in serum prolactin level, mammary tumor virus production, and proliferation of mammary alveolar lesions associated with dietary calorie restriction is responsible for lowering the incidence of mammary tumors in mice.**

Murine mammary tumor virus (MuMTV) is the only RNA tumor virus known to play a major role in the development of mammary adenocarcinoma in mice (1). Other factors such as diet, chemicals, hormones, and ionizing radiation, as well as the genetic, immunological, and physiological status of the host, are known to have a profound influence on the time of onset and the incidence of MuMTV-induced murine mammary tumors (2–4). Dietary calorie restriction has been shown to be one of the most effective ways of reducing significantly the incidence of mammary tumors in C3H mice which are prone to develop breast cancer at a high incidence (5, 6). In these mice, MuMTV is transmitted from the mother to her offspring via the milk (7). It is now known that a reduced calorie intake results in an enhanced cell-mediated immune response (8) and a decrease in the level of circulating anti-MuMTV antibody (9). However, the mechanism by which calorie restriction, MuMTV infection, and other hormonal and immunological functions interact in decreasing the incidence of mammary tumors in C3H mice is unknown.

In this paper, we report that the primary effects of a low-calorie [10 kcal/day (1 kcal = 4.184 kJ)] diet in virgin C3H mice are to decrease the amount of circulating prolactin and the production of MuMTV and to impair the promotion of preneoplastic cells into tumors.

MATERIALS AND METHODS

Diet. Three groups of 4- to 6-week-old female C3H mice weaned at age 4 weeks were obtained from our breeding colony and placed on low fat/high protein diets (differing in the calorific values) for a period of 18–20 months. One group of mice received an unlimited amount of standard Purina laboratory chow ad lib; the daily intake of this group averaged about 20 kcal/day. The second group was fed a standard daily ration of 4.8 g providing 16 kcal. The third group was fed a low-calorie diet providing 10 kcal/day (3 g/day). The source of the diet ingredients, method of diet preparation, and feeding procedures, as well as the housing of animals, have been described (6). The weight of each mouse was recorded monthly, and each mouse was examined at least twice a week to assess general health and to determine whether palpable mammary tumors were present.

Estrus Cycle. To evaluate ovarian function, cells were removed from the vagina with a fine curette, smeared on a slide, and stained with methylene blue (10). Vaginal smears from each mouse were prepared for 19 consecutive days. For simplicity, we considered the pro-estrus, estrus, and met-estrus I as estrogen phases and met-estrus II and di-estrus as luteal phases of the estrus cycle.

Preparation of Serum. Blood from mice fed the laboratory chow or low-calorie diet for about 7–8 months was drawn by capillary tube from the retroorbital plexus, allowed to clot on ice, and centrifuged in a refrigerated centrifuge. Serum was isolated, divided into small portions, and stored at −70°C until used. All serum samples were thawed only once, just prior to testing.

Hormone Measurement. Mouse serum was assayed for thyrotropin (thyroid-stimulating hormone), prolactin, and somatotropin (growth hormone) by double-antibody rat radioimmunoassays using reagents kindly provided by the Hormone

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Abbreviations: MuMTV, murine mammary tumor virus; MAL, mammary alveolar lesions.

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Distribution Program of the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases. The hormones were iodinated with ^{125}I for use as tracers in the radioimmunoassays. The antisera were titered in our laboratory and used at a dilution that precipitated 50% of the immunoprecipitable tracer (11, 12). The hormone preparations that were used for iodination were also used as standards in the assays. Because mouse thyrotropin did not dilute in a fashion parallel to rat thyrotropin in the rat thyrotropin radioimmunoassay, we expressed the amounts of thyrotropin in the mouse serum in ng equiv/ml relative to standard rat thyrotropin.

Electron Microscopy. After removal from mice, the mammary glands were trimmed under a dissection microscope to remove as much fat as possible and then cut into 1-mm cubes. The tissue sections were then fixed in 2.5% glutaraldehyde for 1 hr, postfixed in 1% osmium tetroxide, dehydrated in graded alcohols, and embedded in Epon 812 (13). Tissue sections cut with an LKB microtome were sequentially stained with uranyl acetate and lead citrate (13). Stained sections were examined in the electron microscope for the presence of intracytoplasmic A and B particles (14).

Whole-Mount Preparation of Mammary Glands. Mice were sacrificed by cervical dislocation and the fourth inguinal pair of mammary glands, along with the skin, were fixed in 10% formalin for 5–7 days. The mammary glands were dissected from the skin and were prepared for whole mount or for histopathological examination according to standard methods (15).

RESULTS

Incidence of Mammary Tumors. The effects of various diets on the occurrence of mammary tumors in virgin C3H mice are shown in Fig. 1. Mice were considered to have developed a palpable tumor only after the tumor had reached a size of about 0.5 cm, as measured by calipers. The cumulative tumor incidence in the mice fed lab chow and the 16-kcal/day diet was 62% and 59%, respectively. In contrast, the incidence of mammary tumors in mice fed the 10-kcal/day diet was extremely low (approximately 11%). It is clear from these results that dietary calorie restriction decreases the incidence of mammary tumor development in mice. In an attempt to determine the mechanism by which the diet inhibits tumorigenesis, we studied the influence of the diet on estrus cycle and the hormonal status of the mouse, and on the expression of MuMTV and the development of mammary alveolar lesions (MAL) in the mice.

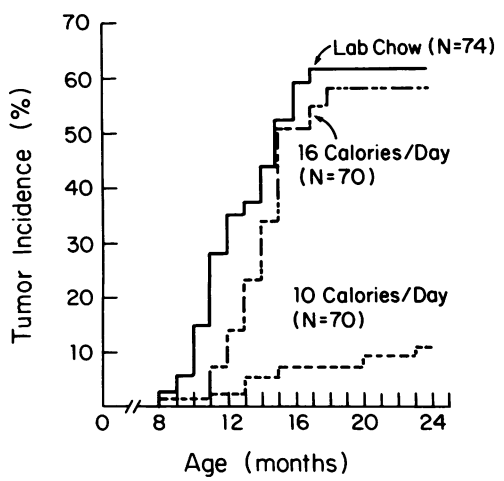

FIG. 1. Mammary tumor incidence in virgin C3H mice fed laboratory chow or defined diets providing 16 or 10 kcal/day. *N*, number of mice examined in each diet group.

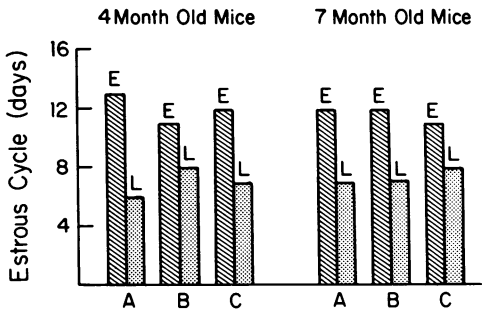

FIG. 2. Estrus cycle of 4- and 7-month-old mice raised on laboratory chow (A) or defined diets providing 16 kcal (B) or 10 kcal (C) per day. Each group consisted of 10–15 mice. Vaginal smears were examined for 19 consecutive days. E, estrogen phase; L, luteal phase.

Estrus Cycle. The changes in ovarian function resulting from calorie restriction were determined by evaluating vaginal smears from individual mice for a period of 19 consecutive days. The estrogen and luteal phases lasted for 11–13 days and 6–8 days, respectively (Fig. 2). The durations of these two phases were similar in all three groups of mice 4 and 7 months old and fed laboratory chow, and 16-and 10-kcal/day diets. These results indicate that calorie restriction does not impair normal ovarian function in mice.

Hormonal Status. The amounts of circulating thyrotropin, prolactin, and somatotropin in 7-month-old mice fed 20 or 10 kcal/day were measured. No significant change was detected in either the thyrotropin or somatotropin levels of C3H mice as a result of caloric restriction (Table 1). However, the level of prolactin was significantly lower in the calorie-restricted mice.

MuMTV Expression. Thin-section electron microscopy of the mammary glands from mice raised on the 16- and 10-kcal/day diets was used to determine if any difference exists in the production of intracytoplasmic A particles (the pronucleocapsids of mature MuMTV) or of budding and mature MuMTV (B particles). Only a few of the mammary cells of mice fed the 10-kcal/day diet occasionally expressed the intracytoplasmic A particles (Fig. 3*a*), and B particles were rarely seen (Table 2). By contrast, most of the mammary gland cells of mice fed the high-calorie diets produced numerous intracytoplasmic A and B particles (Fig. 3 *b* and *c*). These findings suggest that in mice fed the low-calorie diet the synthesis of intracytoplasmic A particles and the assembly of B particles is reduced.

Morphology of the Mammary Glands. Whole-mount preparations of the mammary glands from mice 4–5, 8–9, and 12–13 months old and fed each of the three diets were examined for gross morphological changes and for the presence of MAL. The mammary epithelium of mice of each age group raised on the three different diets was found to occupy the entire area of the fat pad and exhibited a comparable growth of mammary parenchyma, indicating that the dietary restriction used in these studies did not impair the normal growth and development of

Table 1. Thyrotropin (TSH), somatotropin (GH), and prolactin levels in sera of 7-month-old C3H mice on high- or low-calorie diets

Diet, kcal/day	TSH, ng equiv/ml	GH, ng/ml	Prolactin, ng/ml
20	6.2 (7)	21 (14); 23 (12)	7.8 (15); 9.1 (12)
10	6.8 (12)	22 (9); 22 (17)	4.2* (10); 4.4† (12)

Values in parentheses are number of serum samples tested.
* For difference from high-calorie, $P < 0.05$ (unpaired test).
† For difference from high-calorie, $P < 0.02$ (unpaired test).

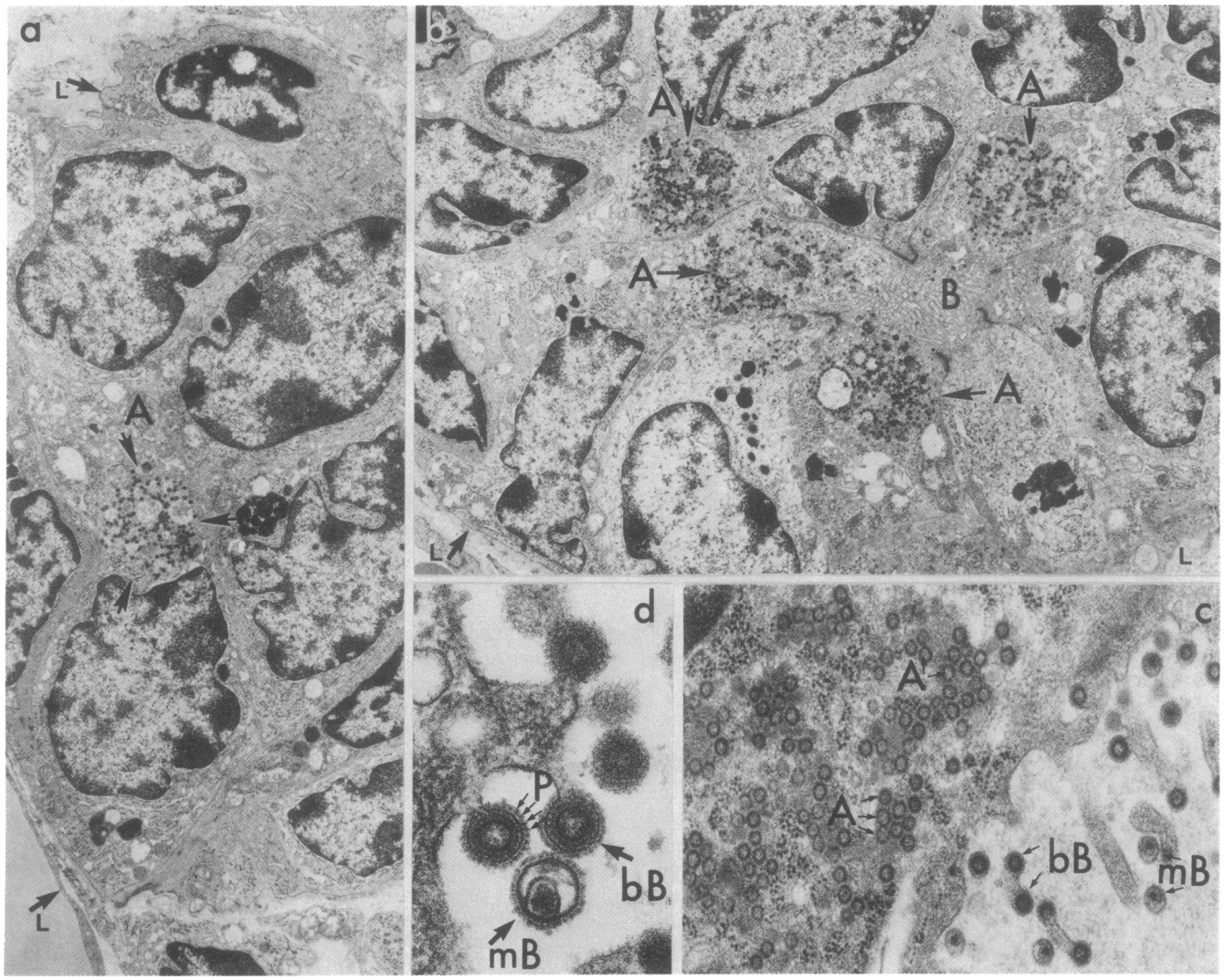

FIG. 3. Electron micrographs of representative mammary gland cells obtained from 7-month-old mice raised on either a 10- (*a*) or 16-kcal/day diet (*b*, *c*, and *d*). *c* and *d* show the structure of type A (A), budding (bB), and mature (mB) B particles. L, edge of a lobule; P, surface projections of MuMTV. (*a* and *b*, ×7,000; *c*, ×30,300; *d*, ×90,200.)

the mammary epithelium. As an example, the morphology of the mammary glands of 11-month-old mice fed the high- and low-calorie diets is shown in Fig. 4 *a* and *b*, respectively. It is difficult to distinguish between MAL and the terminal end buds of small ducts in the whole-mount preparations at low magnification as shown in Fig. 4 *a* and *b* but they can be easily distinguished under high magnification as illustrated in Fig. 4*e*. To confirm the identity of the MAL observed in the whole-mount preparation, sections of the glands were examined after staining with hematoxylin and eosin (Fig. 4 *c*, *d*, and *f*). As shown in low magnification in Fig. 4 *c* and *d*, MAL appear to be irregularly shaped structures which, at high magnification, are clearly seen to consist of numerous lobules (Fig. 4*f*). Comparison of Fig. 4 *c* and *d* shows that the number and size of MAL (thick arrows) in the mammary glands of mice fed the 16-kcal/day diet were greater than those in mice fed the 10-kcal/day diet.

Table 2. Expression of MuMTV particles in C3H mice fed high- or low-calorie diets

Diet, kcal/day	Age of mice, months	MuMTV expression*			
		Type A particles		Type B particles	
16	4–5	2–5+	(12/20)	2–3+	(12/20)
	8–9	3–10+	(14/15)	4–7+	(14/15)
	12–13	5–10+	(20/20)	7–10+	(20/20)
10	4–5	1+	(5/30)	1+	(1/30)
	8–9	1–3+	(4/25)	1+	(3/25)
	12–13	1–3+	(7/20)	1+	(3/20)

* 10+ indicates the presence of numerous particles in the mammary gland cells; 1+ indicates the presence of only a few particles. In parentheses are shown the number of mammary glands with A or B particles/total number of mammary glands examined.

For quantitative purposes, we divided the MAL that we observed into three size classes (Fig. 4*f*): large (L), consisting of a cluster of more than 20 lobules; medium (M), consisting of 10–20 lobules; and small (S), consisting of <10 lobules. The number of MAL observed per mammary gland in the mice fed the laboratory chow and 16-kcal/day diets was greater than that observed in the mice fed the 10-kcal/day diet (Table 3). In mice 4–5 months old, however, there was no difference in the number of small MAL in the groups of mice fed the different diets. In the mice 8–9 months old and fed the laboratory chow or 16-kcal/day diet there were fewer small MAL but more medium and large MAL, suggesting that many of the small MAL present

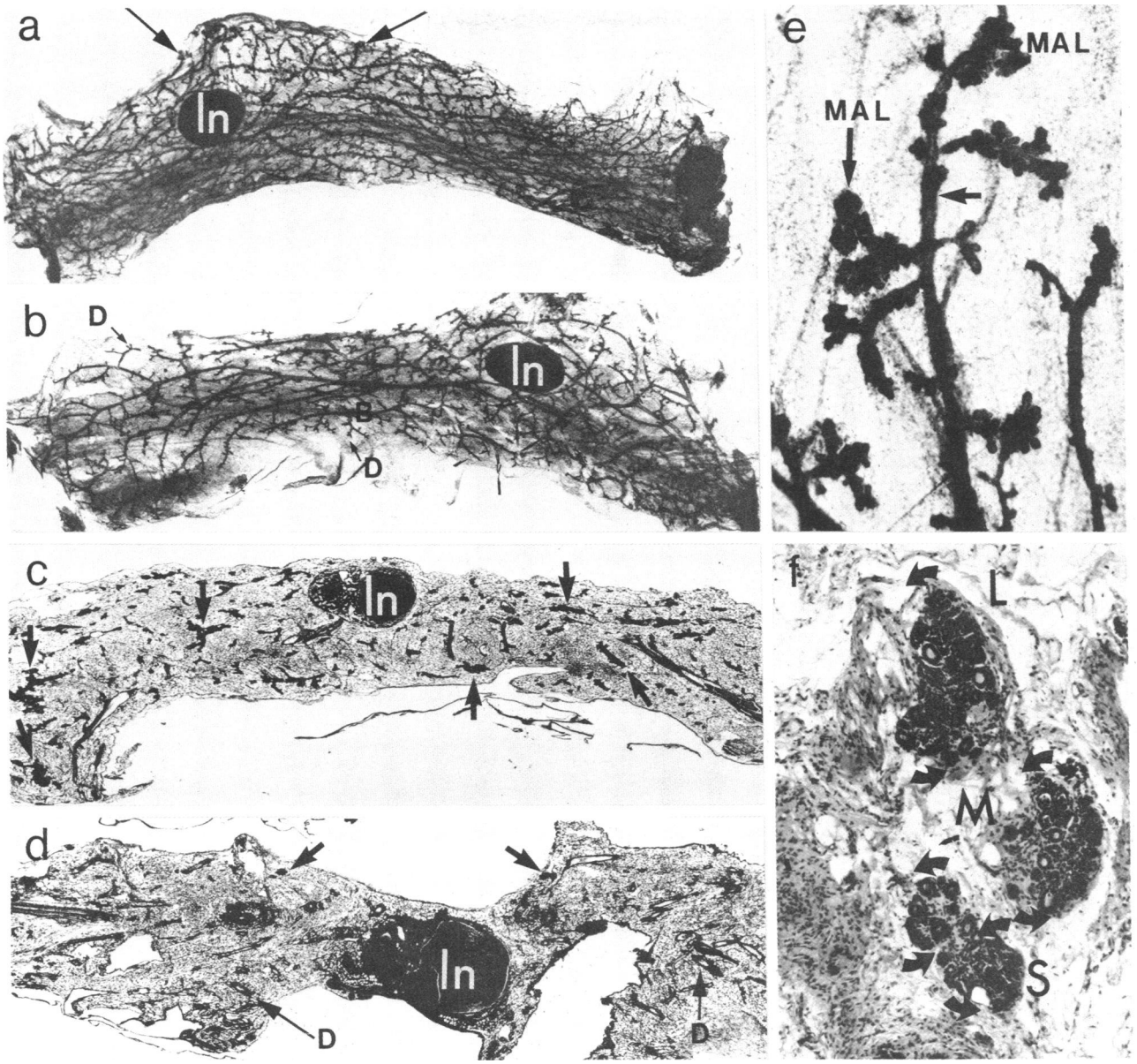

FIG. 4. Morphology of the mammary glands of 11-month-old virgin C3H mice fed different diets. (*a* and *b*) Whole-mount preparations of inguinal (number 4) mammary glands from mice raised on 16 or 10 kcal/day, respectively. The presence of numerous MAL in the predominantly ductal parenchyma (arrow) can be seen in *a*. Sections of mammary glands from mice raised on a diet of 16 kcal/day (*c*) reveal several irregularly shaped MAL (arrows), whereas in mice fed 10 kcal/day there are fewer and smaller MAL (*d*). The ducts in the tissues of these mice can be distinguished from the MAL by their smooth, round, or elliptical appearance. Under high magnification (*e*), the MAL are seen as a cluster of lobules at the tip of the mammary duct (arrow). Sections of mammary glands at high magnification (*f*) reveal that each MAL is formed of clusters of alveoli (arrow) and that the epithelium in each alveolus surrounds a separate lumen. The letters L, M and S designate large, medium, and small MAL, respectively. ln, Lymph node. (*a–d*, ×4.5; *e*, ×32; *f*, ×100).

in the mammary glands of mice 4–5 months old had "progressed" into the larger size classes as the mice aged. This progression did not occur in the mice 8–9 months old and fed the 10-kcal/day diet. A similar, but more pronounced pattern was observed in the oldest (12–13 months) mice. There were many large but few small MAL in the mice fed laboratory chow or the 16 kcal/day diet, whereas the mice fed the 10-kcal/day diet had predominantly small MAL. These results suggest that the dietary calorie restriction prevents the development of small MAL into larger MAL.

DISCUSSION

Several factors, including MuMTV, hormones, and decreased caloric intake, are thought to influence the development of murine mammary tumors. We have found that the incidence of spontaneously occurring mammary tumors in virgin C3H mice raised on a 10-kcal/day diet (11%) was much lower than that of mice fed a 16-kcal/day (58%) or a laboratory chow ad lib (63%). These results confirm previous reports that lowering the calorie intake significantly decreases the incidence of mammary tumors in rodents (5, 6).

The mechanism by which diet influences mammary tumorigenesis is unknown, but prolactin appears to be one of the most significant factors influencing the development of MuMTV-induced mammary tumors in mice and chemical carcinogen-induced mammary tumors in both rats and mice (4, 16, 17). For example, factors that increase serum prolactin (pituitary isografts, median eminence lesions, dopamine blockers) all have

Table 3. Occurrence and size distribution of MAL in virgin young and old C3H mice fed different diets

Age, months	Diet, kcal/day	Occurrence of MAL		MAL, no.		Size distribution of MAL, % of total		
		No.*	%	Total	Per gland	Large	Medium	Small
4–5	20	15/20	75	63	4.2	0	14	86
	16	10/15	66	34	3.4	0	18	82
	10	8/20	40	11	1.8	0	9	91
8–9	20	17/23	74	116	6.8	10	32	58
	16	9/12	75	59	6.6	17	29	55
	10	9/22	41	19	2.1	0	10	90
12–13	20	12/14	85	288	24.0	53	28	19
	16	14/14	100	256	18.3	61	24	15
	10	11/18	61	47	4.3	0	11	89

*Number of mammary glands with MAL/total number of mammary glands examined.

been found to increase the incidence of mammary tumors in 7,12-dimethylbenzanthracene-treated rats (17); pituitary isografts also increase the incidence of mammary tumors and decrease the latent period of tumor development in MuMTV-producing and exogenous MuMTV-free mice (16). Furthermore, MuMTV appears to potentiate the mammotropic effects of drug-induced (18) or isograft-produced prolactin (19). Our present observations also reveal that concentrations of serum prolactin in mice fed a low-calorie diet are significantly lower ($P < 0.05$) than those of mice fed the high-calorie diet, whereas thyrotropin and somatotropin levels are comparable in both groups, suggesting that prolactin plays a role in mediation of the dietary influences on tumorigenesis.

In C3H mice, MuMTV is the major etiological agent of mammary adenocarcinoma (2) and large quantities of type A and B particles are produced by mammary gland cells prior to tumor development (3). Although thin-section electron microscopy is not the method of choice for the quantitation of virus production, it is the only method available to evaluate qualitatively the relative levels of type A or type B particles in mammary gland cells. By examining at least 50 cell sections from each mammary gland, we found that the mammary gland cells of mice fed the low-calorie diet produced A particles less frequently and in far fewer numbers than did the mammary gland cells of mice fed a high-calorie diet (Table 2). In addition, although the mammary gland cells of the mice fed the 16-kcal/day diet produced abundant type A and type B particles, the number of type B particles produced by mammary gland cells of mice fed the 10-kcal/day diet was far fewer than the number of type A particles. The disparity between the production of type A and type B particles in mice fed the low-calorie diet may reflect differences in the expression or processing of the MuMTV gene products. Furthermore, decreased production of type B particles by the mammary gland cells of mice fed a 10-kcal/day diet may be responsible, at least in part, for the low titers of anti-MuMTV antibody in the sera of these mice which we have reported (9). It therefore is possible that the influence of a low-calorie diet on mammary tumorigenesis is mediated by altering at least two factors known to be important in murine mammary tumorigenesis—i.e., prolactin and MuMTV production. These two factors may be interactive, particularly with respect to the development of preneoplastic lesions such as MAL.

MAL are seen in the mammary tissue of all MuMTV-producing high mammary tumor-incidence mouse strains (2), and these lesions have been found to contain more MuMTV particles per cell than do the adjacent normal epithelial tissues (18, 20). Our finding that MAL are markedly decreased in number and in their ability to develop in the mice fed a low-calorie diet compared with mice receiving a higher calorie intake, together with the fact that the mammary gland cells from low-calorie-fed mice contain significantly fewer mature MuMTV particles than do those of high-calorie-fed mice, suggests the existence of a relationship between the decrease in virus production and the decrease in the proliferation of potentially tumorigenic epithelial cells.

Our findings suggest that the interaction among prolactin production, mammary alveolar tissue proliferation, and MuMTV production is profoundly influenced by dietary calorie intake, and that these factors affect mammary tumorigenesis by an as yet unknown mechanism.

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