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Katz, Ellen Bennett, Ph.D.

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THE EFFECT OF DIETARY FAT ON THE GROWTH AND METASTASIS OF THE 13762 MAMMARY ADENOCARCINOMA IN THE FISCHER 344 RAT

by

ELLEN BENNETT KATZ

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1989

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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DEDICATION

To my mother, Dorothy Narwold Bennett, who is always in my thoughts.

To my husband, Ralph, for the love, support, and encouragement he has given me throughout this endeavor.

To my children, Dorothy Ann, Michael Bennett, and Stuart William who are the three reasons this project took a little longer but who are also the center of my life.

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INTRODUCTION

The role of dietary fat in the etiology of breast cancer has been an active and sometimes controversial field of study for over forty years. Since the classic studies of Tannenbaum in the early 1940's (Tannenbaum 1942b) suggested that dietary fat enhanced mammary tumor incidence, numerous animal studies (reviewed by Cohen 1986; Rogers & Lee 1986; Carroll 1987) have clearly shown that dietary fat affects the number and size of chemically-induced mammary tumors, the growth of transplantable tumors, and the appearance of spontaneous tumors in rodents. While laboratory studies point strongly to a high intake of fat as a factor in increasing susceptibility to breast cancer, epidemiological studies are not as conclusive.

EPIDEMIOLOGICAL STUDIES

The most commonly used epidemiological methods for studying the effect of diet on the incidence of cancer are correlational studies, cohort studies, and case-control studies (Lyon et al. 1983). Correlational studies, which measure the relationship between variables at the population or group level, can be international or intranational. International correlation studies show a strong correlation between total dietary fat intake and

mortality from breast cancer (Carroll 1986). Buell (1973) found that first and second generation Japanese women living in the United States and eating a "Western", high fat diet had higher breast cancer rates than their counterparts in Japan. Kolonel et al. (1983) studied different ethnic groups in Hawaii where diet among the different groups varied significantly. They found a strong correlation between high total fat consumption and a higher incidence of breast cancer. The problem with correlational studies is that they do not account for the time when the cancer was induced or determine if the individuals which consume the most fat are the ones actually getting breast cancer.

Cohort studies or prospective studies measure potential risk factors using a large group of healthy individuals (Zaridze et al. 1987). Two recent cohort studies have shown no correlation between dietary fat intake and increased breast cancer risk. Willet et al. (1987) followed a cohort of 89,538 nurses for four years. They concluded that the nurses on what they called a "low" fat diet (an average of 32% of their calories as fat) did not have a reduced risk for breast cancer when compared to nurses on a "high" fat diet (an average of 44% calories as fat). The range of percent of fat content between the diets was very small and could have obscured any effect of dietary fat on breast cancer risk. Another study used the National Health and Nutrition Examination Survey 1 (NHANES

1) Epidemiologic Follow-up Study cohort of 14,407 women aged 25-74 years with an average follow-up period of ten years (Jones et al. 1987). The authors also could find no correlation between breast cancer risk and dietary fat consumption. They did however mention that the intake of dietary fat was "heavily shifted towards relatively high intakes" and this could be a possible explanation for the results (Jones et al. 1987).

Unlike the cohort studies cited above, recent case-control studies have shown a positive correlation between dietary fat consumption and increased breast cancer risk. Case-control studies compare consumption of dietary fat by women with breast cancer to that by women without the disease (Zardize 1987). A study in Israel which included 818 breast cancer patients and two control groups showed a positive correlation between total fat consumption and increased risk of breast cancer (Lubin et al. 1986). An Italian group (Toniolo et al. 1989) studied a cohort of 250 women with breast cancer and 499 women randomly selected from the general population. They concluded that women who ate less than 28% of their calories as fat had a reduced risk for breast cancer as compared to women who derived more than 36% of their calories as fat. There was also a positive correlation with eating less than 9.6% of calories as saturated (animal) fat and a reduced risk for breast cancer. The authors point out that the people of Italy have a large

variability in their diets and contain factions in their populations which eat less red meat than other western industrialized nations and eat more vegetable fats, vegetables and fruits.

Two reviews of breast cancer epidemiology have been published recently (Goodwin and Boyd 1987; Kelsey et al. 1988). The authors of both reviews suggest that since there is such inconsistency in the published reports, further investigation is needed for a more coherent picture of the role of dietary fat in the etiology of breast cancer. Goodwin and Boyd suggest that intervention studies in which a large number of women are asked to modify their diets would answer some of these questions. Unfortunately, the National Cancer Institute has concluded that just such a study would be too costly and not yield enough information to be worthwhile.

EXPERIMENTAL STUDIES

This section details the many experimental studies focusing on the role of dietary fat in mammary tumorigenesis. There are many terms and abbreviations which need to be defined.

There are three main types of fatty acids mentioned. A fatty acid containing no carbon-carbon double bonds ($CH_3(CH_2)_nCOOH$) is a saturated fatty acid. Those containing one double bond are monounsaturated, and those

with 2 or more double bonds are polyunsaturated. Polyunsaturated fats most often encountered are denoted as either n-6 (first double bond starts at the sixth carbon from the terminal methyl group) such as linoleic acid (18:2n-6) or n-3 (first double bond starts at the third carbon from the terminal methyl group). Linolenic acid (18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3), and docosahexanoic acid (DHA, 22:6n-3) are examples of n-3 fatty acids. Fatty acids are found in the diet mainly as triglycerides . Linoleic and linolenic fatty acids cannot be synthesized by humans and must be obtained from plant sources and are therefore called essential fatty acids (EFA) (Zubay 1983).

Many types of fat were used in the diets of the animals in the experimental studies to be discussed. The following abbreviations will be used for the types of fat most frequently mentioned: corn oil (CO); olive oil, mainly monounsaturated oleic acid (OO); beef tallow, mainly saturated fat (BT); menhaden fish oil, mainly polyunsaturated fat, n-3 (MO).

When the percentage of fat is given for a diet, it will mean the weight of the fat compared to the total weight of the diet (wt/wt). A high fat diet (HF) will denote a diet with 20% or more of its weight as fat (i.e., HFCO is a diet containing 20% corn oil). A low fat (LF) diet contains 5% or less fat (i.e., LFOO is a diet of 5% olive oil).

Unlike the epidemiological studies, controlled laboratory experiments consistently show a strong correlation between the quantity and quality of dietary fat and mammary tumorigenesis. The development in the early 1960's of the dimethyl-benz(a)anthracene (DMBA) model for chemically inducing mammary tumors and the more recent use of N-nitrosomethylurea (NMU) stimulated research on the effect of diet on mammary tumorigenesis (Carroll 1986). Carroll and Khor (1971) showed that both the quantity and type of dietary fat were important factors in determining the number of mammary tumors arising in the Sprague-Dawley rat after exposure to a single 50 mg. dose of DMBA. The rats were fed semi-purified diets containing 10% and 20% w/w corn oil ("high" fat diets) and 0.5% or 5% w/w corn oil ("low" fat diets) to assess the influence of the quantity of fat had on tumor number. Diets containing 20% by weight of different fats (polyunsaturated fats - corn, soybean, sunflower, cottonseed oils; monounsaturated fats - olive and rapeseed oils; saturated fats - coconut oil, butter, tallow, lard) were used to determine the effect of the quality of fat on tumor incidence and number. Carroll and Khor found that animals on a "high" fat diet developed significantly more mammary adenocarcinomas than those on a "low" fat diet. In addition, polyunsaturated fats enhanced the yield of adenocarcinomas as compared to saturated fats.

One of the problems of these early studies is that the essential fatty acids, especially linoleic acid, varied widely among the diets and the saturated fat diets were especially deficient in these fatty acids. Consequently, Hopkins et al. (1981) performed DMBA experiments using different oils supplemented with corn oil to ensure an adequate amount of essential fatty acids for mammary tumor growth. Their results indicated that once the EFA requirement was met, the additional fat effect on tumor incidence and growth seemed to be independent of the type of fat. However, saturated and monounsaturated fats did not promote the growth of the primary tumors as well as the polyunsaturated fats.

Ip et al. (1985) have taken these studies further and shown that in rats fed a 20% fat diet, incidence of mammary tumors after DMBA administration depends on the percent of EFAs in the diet not the type of fat. They found that a diet containing 3-4% by weight EFAs was required for maximum tumor incidence.

The question of at what point different fats affect the process of chemically-induced tumorigenesis has been studied by many investigators. Rogers et al. (1983) fed a high fat (HF, 20-25%) lard diet (mainly saturated fat) to rats either before DMBA administration only, or before and after DMBA treatment, or throughout the entire experiment. Rats taken off the HF lard diet, were switched to the LF lard diet. Another group of rats was given a low fat (LF,

4%) lard diet throughout the experiment. They showed that all HF groups developed mammary tumors significantly earlier than the LF group. Also, feeding a HF lard diet only before DMBA treatment enhanced tumorigenesis to the same extent as feeding the HF diet to the animals before and after the treatment. Rogers et al. suggested that these results showed lard influencing mammary gland carcinogenesis at initiation rather than the promotional phase.

More recently, Rogers et al. (1986) administered DMBA subcutaneously to avoid any problem with absorption of the DMBA which might accompany the consumption of a HF lard diet. In contrast to their previous results, a HF lard diet did not significantly enhance tumorigenesis. Also, when DMBA was given by gavage, the only groups to show significant enhancement of tumorigenesis were those consuming the HF lard diets throughout the entire experiment or from the time of DMBA adminstration. Rogers et al. could not explain why, in these experiments, lard seemed to have no influence at initiation.

Kritchevsky et al. (1984) found in their experiments that high saturated fat diets acted at both initiation and promotion of tumors induced by DMBA. They fed Sprague-Dawley rats a high fat (19% , mainly coconut oil) diet prior to DMBA ingestion, then kept them on either the high fat diet or put them on an isocaloric low fat (3.9%) diet. Both groups developed twice as many tumors as the

rats fed low fat diets before carcinogen administration. They suggested that these results indicated that a high fat diet can "affect initiation and/or be cocarcinogenic in DMBA-induced mammary tumorigenesis" (Kritchevsky et al. 1984).

Dao and Chan (1983) used corn oil diets (polyunsaturated fat - PUF) to study the effect of the duration of a high fat diet on chemically-induced tumorigenesis. They used the direct acting, water soluble NMU to induce mammary tumors in Fischer rats. They found, as in the DMBA experiments, that feeding the animals a HF diet throughout the experiment resulted in a higher incidence of tumors as compared to the LF group and the group receiving the HF diet only after DMBA treatment. Feeding a HF diet after carcinogen administration promoted mammary tumor growth. Dao and Chan also showed that the duration of a HF PUF diet affected the enhancing ability of the HF diet. The HF diet fed to the rats four weeks before or after NMU treatment had no effect on tumor incidence. However, if the feeding time were extended to six weeks or after initiation, the tumor incidence rose proportionately with the number of weeks fed.

Chan et al. (1983) also used the NMU model in Fischer rats to study the effect of different types of fat on tumorigenesis. The rats were fed LF corn oil, HF corn oil, HF lard, HF beef tallow, or HF coconut oil and mammary tumor incidence was assessed 28 weeks after a

single dose of NMU. The incidence of mammary tumors was highest in the HF corn oil group (85%) and lowest in the LF corn oil group. The incidence of mammary tumors in the HF corn oil rats was greater than in the other HF groups (HF lard, 65%; HF beef tallow, 50%; HF coconut oil, 43%). There was a positive correlation between the total amount of linoleic and oleic acid in the diet and the incidence of mammary tumors. Chan et al. concluded that the total amount of these fatty acids was the most important feature of the diets affecting the incidence of mammary tumors induced by NMU.

To test this hypothesis further, Cohen et al.(1986), also using the NMU model, tested oils varying widely in the amounts of linoleic and oleic acids. Tumor incidence in animals fed oils high in linoleic acid as compared to oleic acid (corn oil, safflower oil) was significantly greater in the rats fed oleic acid rich oils (olive oil, coconut oil).

Recently, the effect of fish oils on both NMU and DMBA induced mammary tumorigenesis has been investigated. These oils contain other types of polyunsaturated oils (n-3) and low levels of linoleic acid (n-6). Jurowski and Cave (1984) fed female Buffalo rats 0.5%, 3%, and 20% menhaden oil diets after treatment with NMU. All animals were killed when their tumors were greater than 2 cm. The 20% MO group had significantly fewer and lighter tumors when compared to the 5% MO animals. The average age of

death was significantly increased in the 20% MO diet group when compared to the 5% MO group. The authors suggest that these results indicate that menhaden oil reduced tumor growth. O'Connor et al. (1986) fed rats from weaning diets containing either menhaden oil or corn oil at the 20% wt/wt level. At 50 days, 5 mg of DMBA was given. The rats on the menhaden oil diet developed significantly fewer mammary tumors than the animals on the corn oil diet.

The effect of dietary fat on the growth of transplantable tumors has also been well studied. Hillyard and Abraham (1979) tested the effect of a 15% corn oil diet and a fat free diet on the growth of six different mouse and rat mammary adenocarcinomas. The mice and rats were maintained on nutritionally complete stock diets until implantation of the tumor piece. After implantation, the animals were switched to either the 15% fat diet or the fat free diet. In all cases, the animals on the the 15% corn oil diet developed tumors of significantly greater mass than the tumors of animals on a fat free diet. When as little as 0.1% linoleic acid was added to the fat free diet, two of the transplantable tumors in the mice grew just as well as the tumors in the 15% fat group. However, two other tumors types in the mouse and the R3230AC tumor in the Fischer 344 rat still did not grow as well in the animals on fat free diets supplemented with 0.1% linoleic acid when compared to the

tumor growth in animals on the 15% corn oil diet.

Gabor et al. (1985) investigated the difference in growth rates of a transplantable tumor in BALB/c mice on either a 10% corn oil diet or a 10% hydrogenated cottonseed oil diet containing no linoleate. The corn oil diet promoted tumor growth significantly more than the cottonseed oil diet. Ten days after tumor implant, mice were injected i.p. with 50 uCi[^{3}H]d thymidine. At different time intervals, the mice were sacrificed and their tumors excised and fixed. The tumor was then sectioned and covered with photographic emulsion. The grains of radioactivity were counted on the developed slides. Tumor cell loss was calculated and determined to be greater in the linoleate free animals accounting for the slower tumor growth rate seen in these animals.

Gabor et al. (1986) also studied the effect of different types of fat on the growth of mammary adenocarcinoma IX. They found that a 10% menhaden oil (MO) or 10% hydrogenated cottonseed oil (HCTO) diet significantly reduced the average size of the tumors when compared to the tumors in the 10% corn oil (CO) group. When diets with a 10% fat level contained nine times as much MO as CO, tumor growth was inhibited when compared to tumor growth in animals on a 10% CO diet. This inhibition of growth did not occur in animals who ate diets containing nine times as much HCTO as CO.

Using the DMBA-4 transplantable mammary tumor in

Wistar-Furth rats, Kollmorgen et al. (1983) concluded that tumor growth was significantly greater in rats fed a 10 or 20% corn oil diet than in rats fed a 2 or 5% corn oil diet. Boylan and Cohen (1986) and Katz and Boylan (1987a), however, found no significant difference in the growth of the 13762 mammary adenocarcinoma in Fischer 344 rats on either a 23% or 5% corn oil diet. This was true of the tumor growth in both virgin 8 week old animals and in retired breeders (10-12 months). High fat beef tallow (20%) and high fat olive oil (20%) diets containing about 3% linoleate promoted growth of the 13762 mammary tumor as well as the high fat corn oil diet (Katz and Boylan, 1989a).

The effect of omega-3 fatty acids from fish oil on transplantable tumor growth has also been studied. Oza and Karmali (1986) found that the growth of the R3230AC mammary tumor was significantly inhibited in rats fed a diet rich in fish oil (23.5%) when compared to tumor growth in animals fed a 5% fish oil diet. Kort et al. (1987) fed BN/Bi rats diets rich in saturated fatty acids (cacao butter), unsaturated fatty acids of the n-6 type (safflower oil), and unsaturated fatty acids of the n-3 type (menhaden fish oil). They found the diet containing the n-3 oil significantly inhibited the growth of the BN473 mammary tumor as compared to the growth of the tumor in the animals on the other types of diets.

Spontaneous mammary tumor incidence and growth in

rodents has also been shown to be influenced by dietary fat. Gridley et al. (1983) found that mice fed an ad libitum low fat (corn oil or butter) diet had the lowest spontaneous mammary tumor incidence when compared to animals on a high fat diet. Unlike the low fat mice, all of the mice on the high fat diets developed mammary tumors. Kort et al. (1985a) fed Brown Norway virgin female rats diets high or low in linoleic acid throughout their lives. They found that while the incidence of tumor-bearing rats did not increase when the two groups were compared, the number of mammary tumors found in the high linoleic acid group was significantly greater than in the low linoleic acid group, because of high tumor multiplicity. Finally, Silverman and Kent (1987) fed mice 20% and 5% corn oil diets throughout their lives. The animals on the 20% corn oil diet developed mammary tumors first at 199 days compared to the first tumors in the 5% corn oil group at 270 days.

MECHANISMS OF ACTION

Several mechanisms have been proposed to explain how dietary fats influence initiation and promotion of tumorigenesis. Dietary lipids can affect cell membrane structure , prostaglandin synthesis , cell turnover and differentiation, hormone levels in the blood, and the immune system. Dietary fat is also a concentrated source

of calories. Some investigators feel that this increased caloric intake with the consumption of a high fat diet is the primary way in which dietary fat affects tumorigenesis. Affecting these structures and processes could in turn affect tumorigenesis in a variety of ways.

Modifying the amount and/or type of lipid in the culture medium of cultured cells or in the diet of animals affects the composition of the cell membrane. Dietary modifications in humans have produced alteration in human erythrocytes and platelets (Ahmed 1984; Farquhar 1963). These alterations in the membrane can affect membrane bound enzyme systems, neurotransmittor uptake in the brain, and B adrenergic receptor binding (Spector 1985).

The amount and type of fatty acids in the diet also affect the fatty acid composition of mammary gland adenocarcinoma cell membranes. Rao and Abraham (1975) analyzed the composition of total fatty acids in a serially transplanted adenocarcinoma in C_3H mice which were fed either a fat free diet or a diet containing 4% fat. They found that the total fatty acid content of the tumor was very similar in both diet groups, but that the tumor in the mice fed 4% fat diets contained 8 times as much linoleate as the tumors of the mice fed the fat free diet. Hubbard and Erickson (1987) investigated the differences in the fatty acid composition of the 4536 mouse mammary tumor when the animals were fed high fat diets differing in linoleic acid content. At each level

of increase of linoleic acid in the diet, the level of linoleic acid in the tumors increased significantly. Oleic acid (18:1), lauric acid (12:0), myristic acid (14:0), and palmitoleic acid (16:1) decreased in the tumor tissue with increasing levels of linoleic acid in the diets.

Prostaglandin synthesis can be affected by dietary modifications (Spector 1985). Prostaglandins are synthesized from arachidonic acid (20:4n-6). Consumption of an increased amount of linoleic acid (18:2n-6), the precursor for arachidonic acid, leads to increased arachidonic acid levels in some tissues (Mathias and Dupont, 1985). This could lead to increased release of prostaglandins when the cell receives appropriate stimuli. Prostaglandin E_2 (PGE_2) synthesis by mononuclear cells of the spleen of rats fed a high polyunsaturated fat diet is increased (Kollmorgen 1983).

Another mechanism proposed for the way dietary fat might affect carcinogenesis is by influencing cell turnover and differentiaton of the mammary gland. Under normal dietary conditions (low fat), Russo et al. (1983) have shown that mammary gland susceptibility to chemical carcinogens is decreased with decreased DNA synthesis and increased differentiation. Lee and Rogers (1983) found high fat lard diets caused an increase in DNA synthesis while differentiation, assessed by grading whole mounts of the mammary gland, appeared slightly retarded, providing a possible explanation for the effect of a HF lard diet on

DMBA-induced mammary tumorigenesis. King et al. (1983), also by grading whole mounts, found that rats given a high polyunsaturated fat diet (20%) had at 50 days of age more terminal end buds and alveolar buds (areas of proliferation), which are potential target sites for carcinogens. Low polyunsaturated fat diets (5%) resulted in more differentiated glands at 50 days of age than rat chow animals.

Modulation of the amount of hormones circulating in the body could be another way high fat diets affect tumor growth. In post-menopausal women, most estrogen is produced by the aromatization of androstenedione in adipose tissue (Longcope 1978). Estrogen is thought to have a permissive role in tumorigenesis, increasing cellular proliferation in target tissues. Consuming a high fat, high calorie diet would increase the amount of adipose tissue found in the body and the amount of androgen aromatized to estrogen. This increased level of estrogen results in stimulation of target tissues such as the breast and endometrium in post-menopausal women where such stimulation is usually lacking.

Chan et al. (1977) proposed that high fat diets increased prolactin levels, but not estrogen levels in rats. They found that high fat diets increased the ratio of prolactin to estrogen in the circulation. However, Aylsworth et al. (1984) could not find an increase of prolactin in rats fed high fat diets. The increase that

Chan et al. observed could have been brought about by the use of ether to anesthetize the rats in order to take their blood. Ether stimulates the secretion of prolactin. Furthermore, when Aylsworth et al. either increased or decreased prolactin or estrogen in the circulation, by chemical or surgical means, the effect of the high fat diet remained the same. Likewise, when Sylvester et al. (1986) fed rats low corn oil (5%), high corn oil, high beef tallow, high lard, and high palm oil diets (all 20%) they could find no differences in the basal or surge levels of serum prolactin, luteinizing hormone, or estradiol. Thus, a HF diet does not seem to be affecting mammary tumorigenesis by increasing the amount of prolactin.

Another mechanism proposed for the influence of dietary fat on tumorigenesis is by modulation of the immune system (reviewed by Erickson 1986; and Johnston 1985). If the immune response is suppressed, its role of stopping or retarding neoplastic growth would be diminished. Some investigators have found that a high polyunsaturated fat diet is immunosuppressive (Mertin 1977). As mentioned before, a high polyunsaturated fat diet can lead to increased production of prostaglandins. PGE_2 in physiological concentrations has been shown <u>in vitro</u> to inhibit many immune parameters. PGE_2 added <u>in vitro</u> suppresses generation of cytotoxic cells in mixed lymphocyte cultures (Darrow 1980) and inhibits natural

killer cells (Brunda 1980).

Thomas and Erickson (1985) studied the effect of low, moderate, and high saturated fat (SF) and polyunsaturated fat (PUF) diets on the function of cytotoxic T cells. Mice were fed diets containing no EFA, 0.5% EFA, 8% or 20% PUF, and 8% or 20% SF. C57BL/6 mice on these diets were immunized with cell line 168 tumor cells. Peritoneal exudate cells were collected at 10 days to determine a primary response and at 15 days after a second injection of tumor cells. These cells were tested in a five hour cytotoxicity assay with the 168 tumor cells, labelled with radioactive chromium. The authors found that the primary cytolytic response was significantly reduced in mice fed both the SF and PUF diets at 8 and 20% compared to the EFA control diet. The secondary response was also reduced in 8 and 20% SF and 20% PUF groups.

Leung and Ip (1986) investigated the effect of a high PUF diet on the ability of splenic natural killer (NK) cells from the Sprague-Dawley rat to kill a YAC-1 lymphoma target cell. NK activity was suppressed in the high fat (20%) group as compared to the low fat (5%) five days after the initiation of the diet. However, no difference in NK activity between the two groups was seen at any of the later time periods tested.

Tiwari et al. (1988) studied the effect of a high and low PUF diets on the composition of the cell membranes of the T and B lymphocytes in both young and old mice. They

reasoned that if the cell membrane structure were affected by diet and age, cell membrane mediated events in the immune system would also be affected. The maximal change in the cell membranes of the young animals occured 4 to 6 weeks after initiation of the diets. The young animals fed the high PUF diet for this time period had a higher level of linoleic acid in their T cell membranes when compared to old animals fed the diet for the same time period.

Finally, many investigators believe that the enhancement of mammary tumorigenesis by dietary fat may be due to the caloric density of a high fat diet and therefore the increased calorie consumption of animals on a high fat diet. Kritchevsky et al. (1984) fed rats high (19.4%) and low (3.9%) fat coconut oil diets supplemented with 1% corn oil or diets that were low fat, high calorie (more sucrose added), or high fat, low calorie (less casein) diets. They also pair-fed rats 60% of the calories of the ad libitum-fed low fat animals. The restricted diet contained the same minerals and fiber as the ad libitum diet but the restricted diet rats received twice as much fat (8.9 vs. 3.9%). The rats in the high fat group had a higher tumor incidence than the low fat group. However, the low fat, high calorie animals had a higher tumor incidence than the high fat, low calorie rats. Also the restricted calorie diet animals developed no tumors after DMBA administration. Kritchevsky et al.

suggest that their results indicate that caloric intake may have a greater influence on tumorigenesis than dietary fat.

Thompson et al. (1985) also compared NMU induced mammary tumor incidence and yield in animals on various ad libitum and controlled calorie diets. The tumor yield in the high fat rats fed ad libitum was significantly greater than that of the low fat group. The other high and low fat groups were fed the same net utilizable energy of 50 kcal per rat per day from day 1 after NMU treatment to day 17 and from then fed ad libitum. There was no difference in tumor yield and incidence between the high and low fat groups on the diets with equal utilizable energy.

Boissoneault et al. (1986) also examined the effect of feeding the same net utilizable energy in high fat and low fat diets on DMBA induced tumorigenesis. Rats were fed a high fat corn oil diet (30%), low fat corn oil diets (5%), or high fat diets restricted to 89% of the calories consumed by the low fat animals fed ad libitum. Their rationale for this restriction was that Boutwell et al. (1949) had shown that a 30% fat diet was utilized 11% more efficiently than a 5% fat diet in young rats. Tumor incidence was 73% in the high fat ad libitum group, 43% in in the low fat ad libitum group and only 7% in the high fat restricted group. The authors suggest that these results indicate that it is not just the percentage of fat in the diet but "rather a complex interaction involving

energy intake, energy retention, and body size" which affects tumor incidence.

Also using the NMU model, Cohen et al. (1988) restricted the calories of high fat and low fat animals to 75% of the calories consumed by comparative ad libitum fed animals. The high fat restricted group had a tumor incidence of 8% compared to an incidence of 78% in the high fat ad libitum group. They also included in the experiment a group of high fat ad libitum fed animls which had free access to an exercise wheel. These rats had a tumor incidence (63%) lower than that of the high fat sedentary rats and similar to the low fat sedentary rats fed ad libitum (58%). Cohen et al. also found that the active rats consumed more food and gained more weight but had less body fat than the sedentary groups. They suggest that their results which indicated a "promoting effect of HFAL (high fat ad libitum) diets and the antipromoting effects of energy restriction-increased energy expenditure" showed no correlation with either "body weight, caloric intake, or the ratio of fat to lean body mass" pointing to the possibility that these effects might be "mediated indirectly and, possibly, independently via paracrine, endocrine, or neurohormonal mechanisms."

From the above discussion, it is evident that much is known about the effects of dietary fat on the growth of mammary tissue and the initiation and promotion of mammary

gland tumors in animal models. In contrast, until recently, few studies had been done on the influence of dietary fat on metastasis from mammary tumors. Since deaths from breast cancer occur primarily from the metastatic spread of tumor cells mainly to lung, brain, liver, and kidneys (Hellman, 1982), information from human and animal studies on the influence of fat on metastasis would be very valuable. Gregiorio et al. (1985) analyzed information obtained from 2,110 patients admitted with breast cancer to Roswell Park Memorial Institute from 1957 to 1965. They showed that, in women with distant disease, total fat intake was significantly related to survival. The relative risk of death increased 1.44 fold for each additional 1000 grams of fat consumed monthly.

Before the beginning of this thesis project, there were few animal studies pertaining to the influence of dietary fat on metastasis. Kollmorgen et al. (1983) studied the effect of corn oil diets on the spread of the DMBA-4 transplantable tumor. They found that the regional lymph nodes were larger in tumor bearing rats on 10% or 20% corn oil diets than in rats fed 2% or 5% fat. There were many limitations to this study including the fact that the tumors were implanted when the rats were less than 1 month old and the rats were on the diet for a very short time.

Gabor (1985) fed mice diets of 10% corn oil or 10% hydrogenated cottonseed oil containing no linoleate. The

mice were implanted with a metastasizing mammary tumor and their lungs checked for metastatic lesions at 2, 3, and 4 weeks after implantation. The number and size of the lesions in the lungs of the corn oil fed animals was significantly greater than those found in the mice fed the hydrogenated cottonseed oil diet.

Kort et al. (1985b) fed BN/Bi weanling rats high fat diets rich in menhaden oil or lard. After nine weeks on the diets, the 12 week old animals were inoculated with the BN472 transplantable mammary adenocarcinoma. The animals were sacrificed 31 days later and lung metastasis scored semi-quantitatively by counting metastasis in one 3 m section of the left lung. Kort et al. could find no significant difference in lung metastasis between the two diet groups.

Boylan and Cohen (1986) showed that in Fischer 344 rats implanted with the 13762 tumor at 9 weeks of age there was no difference in the extent of pulmonary metastasis between animals fed the high corn oil (23%) or low corn oil (5%) diets. They pointed out that these results were consistent with results of epidemiological studiets showing that there is no increase in survival in pre-menopausal Japanese women compared to American women on higher fat diets (Sakamoto et al. 1979). The rats they used were young, cycling animals analagous to the pre-menopausal state in women. Since there was an increased survival rate in post-menopausal Japanese women

as compared to American women, they suggested that a difference in metastases might be seen using older animals as the host for the tumor.

The following chapters of this thesis are a direct result of this suggestion. The experiments in Chapter 1 investigate the effect of low and high polyunsaturated diets on the primary tumor growth and extent of pulmonary metastasis from the 13762 mammry tumor in both intact and ovariectomized retired breeders. Chapter 2 contains experiments designed to answer the question of whether changing the diet at tumor implant might affect primary tumor growth and metastasis and whether starting the diets at implant would have the same influence as pre-feeding the diets then implanting the tumor in retired breeders. Chapter 3 includes experiments which compared the extent of metastasis from the 13762 MT in retired breeders fed diets containing different types of fat (corn oil, beef tallow, and olive oil). Finally, the effect of restricting calories (to 70% of the normal amount consumed by retired breeders) on primary tumor growth and pulmonary metastasis in retired breeders fed high and low corn oil diets is the subject of Chapter 4.

Chapter 1

THE STIMULATORY EFFECT OF HIGH POLYUNSATURATED FAT DIET ON LUNG METASTASIS FROM THE 13762 MAMMARY ADENOCARCINOMA IN FEMALE RETIRED BREEDER RATS

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ABSTRACT

The effect of a high-fat (HF) diet (23% corn oil) on the growth and metastasis of the 13762 mammary tumor in Fischer 344 retired breeder (RB) and young virgin (YV) female rats was studied. The RB (10-12 mo old) and YV (8 wk old) rats were fed the HF or low-fat (LF) diet (5% corn oil) prior to and following tumor implantation for a total of at least 10 weeks. The growth rate of the primary tumor in the intact RB and YV was not affected by the HF diet. In RB rats ovariectomized 4 weeks prior to tumor transplantation, the tumor grew significantly faster in the HF group as compared to the LF group. The total volume of metastatic tumor nodules in the lungs of the HF goups was significantly higher than that in the lungs of the LF groups in both the intact and ovariectomized RB. In the YV, there was no difference in pulmonary metastatic burden between the HF and LF groups. The weights of the HF intact and ovariectomized RBs were higher than those of the LF animals. However, when pulmonary metastatic tumor burden was compared to body weight at implant or sacrifice, there was no significant correlation in either the HF or LF groups. These results suggest that an HF diet enhance the growth of pulmonary metastases in the intact and ovariectomized RB but not the YV rats and that the effect of the HF diet on pulmonary tumor burden cannot be attributed entirely to increased body weight.

INTRODUCTION

The significant risk of death from metastasis after treatment of primary breast cancer even in otherwise asymptomatic women has stimulated research for adjuvant treatments including chemotherapy and dietary interventions (Harris et al. 1985; National Institutes of Health, 1986). There is substantial epidemiological and experimental evidence implicating the type and levels of dietary fat in the initiation and growth of primary breast cancer in humans and animals (Cohen 1986; Rogers et al. 1981; Carroll et al. 1983; Hillyard & Abraham 1979; Miller 1986). Polyunsaturated fats such as corn oil are particularly effective in promoting the growth of rodent mammary tumors (Hopkins et al. 1981; Ip et al. 1985). However, there are few studies showing an association between dietary fat and breast cancer metastasis. Perhaps the most direct epidemiological evidence in humans comes from Gregorio et al. (1985) who did an historical prospective study to determine whether the amount of fat in the diet affected the progression of disease and survival of women with breast cancer. They found that the estimated risk of death increased 1.4 fold for each 1000g increment in the monthly fat intake. Indirect evidence comes from cross-sectional studies comparing survival rates of Japanese and American breast cancer patients, where, even when matched for stage at diagnosis, postmenopausal Japanese women showed an

improved rate of survival (Sakamoto et al. 1979; Moolgawkar et al. 1979). Given the known differences in diet between these two populations, these data have been used in support of a role of dietary fat in the progression of breast cancer in postmastectomy breast cancer patients. Several other groups have studied the association between obesity (or body mass) and prognosis (Miller 1986; Kirchner et al. 1982; Brisson et al. 1984). Whereas there are generally positive associations, it is unclear whether obesity *per se* is an adequate measure of a high dietary fat consumption.

Turning to animal models, Kollmorgen et al. (1983) studied the effect of corn oil diets on the spread of the DMBA-4 transplantable mammary tumor. They found that pre-pubertal tumor-bearing rats fed a high fat (10% or 20%) diet had larger lymph nodes than rats fed a normal fat diet (2% or 5%) at the time of sacrifice. No data on distant organ metastases were presented. Erickson et al. (1984) also studied the effect of different fat diets on experimental metastases of the cell line 168 mammary tumor in the BALB/c mouse. They found that the configuration of the fat (*trans* vs. *cis*), but not the amount fed, affected the survival of injected radiolabeled tumor cells in distant organs. There were fewer tumor cells found in the liver and spleen of mice on 20% *trans* fatty acid diets than in mice fed diets containing a high level of *cis* fatty

acids. Likewise, there were fewer viable tumor cells in the livers of mice fed the 5% trans diet than the 5% cis corn oil diets. The capacity of the disseminated tumor cells to grow in these distant sites was not assessed.

Taken together, the epidemiological and experimental animal data have encouraged clinical researchers to attempt dietary intervention trials aimed at lowering the amount of fat in the diet to 20% of caloric intake in two groups of women: those at high risk for developing breast cancer and those with proven stage II breast cancer as a means of secondary prevention (National Institutes of Health 1986). While this represents an important step forward, it will be difficult to test in clinical trials the many possible permutations of interest: variation in amount and type of fat, in the receptor status of the primary tumor, in the endocrinologic state and age of the host, etc.

Consequently, we chose to study the effect of dietary fat on breast cancer metastasis using one of the few metastasizing rodent mammary tumor systems, the 13762 mammary adenocarcinoma in the Fischer 344 rat (Bogden et al. 1974; Neri et al. 1982). Our preliminary studies using young virgin female rats failed to detect any significant difference in either lung metastatic burden or regional lymph node enlargement between groups on a 5% and 20% corn oil diet (Boylan & Cohen 1986). These results in young

virgin rats are consistent with the epidemiological evidence of Sakamoto et al.(1979) who observed that premenopausal Japanese and American women exhibited the same survival patterns. Since postmenopausal Japanese breast cancer patients were found to have an improved prognosis compared to American women with breast cancer, we chose to use intact and ovariectomized retired breeder female rats as tumor hosts to simulate a "peri/post-menopausal" condition. In this paper, we demonstrate that both intact and ovariectomized retired breeder females fed a high fat diet for at least 4 weeks before and 6 weeks after tumor implantation had a significantly greater volume of lung metastases at the time of sacrifice.

MATERIALS AND METHODS

Animals and Diets. Female Fischer 344 retired breeders, age 10-12 months, and young virgins, age 4 weeks, were purchased from Charles River Breeding Laboratories, North Wilmington, MA. They were housed three to a cage in a temperature and light controlled room and allowed to acclimate for a week before being fed the special diets ad libitum. The animals were divided into two groups in each experiment: one group of animals received a high fat diet (23% corn oil) while the other group received a low fat diet (5% corn oil). It should be noted that a "low" fat diet contains the same amount of fat as the "normal" commercial rat chows. These semi-purified diets were obtained from Bio-Serv Inc., Frenchtown, NJ, in pellet form, stored in the dark at 4°C, and used within 4 weeks. Composition of the diets is shown in Table 1. To confirm that rats in the high and low fat groups consumed approximately equivalent calories when fed ad libitum , the amount of food eaten by 6 animals from each dietary group housed 3 to a cage was determined by calculating the weight of food consumed by each cage over a 24 hour period; this was repeated daily for 6 days.

Tumor Implantation. Two mm^3 pieces of the transplantable 13762 mammary adenocarcinoma were implanted subcutaneously into each rat anterior to the 4th nipple. The tumor was obtained through Dr. A. Bogden, Mason

Table 1. Composition of Semi-Purified Low Fat and High Fat Corn Oil Diets

	Composition (%)	
	Low Fat Diet	High Fat Diet
Ingredients		
Dextrose and Maltose Dextrin	35.20	16.90
Corn Starch	23.80	23.80
Casein	23.90	23.90
Corn Oil	5.00	23.00
Alphacel	5.90	5.90
Mineral Mix[a]	4.13	4.13
Vitamin Mix[a]	1.77	1.77
DL Methionine	.30	.30
Choline Bitartrate	.24	.23
Energy Value	3.68 kcal/g	4.59 kcal/g

[a] AIN-76 Formula

Research Institute, Worcester, MA. from the Animal and Human Tumor Bank supported by the Breast Cancer Program, N.C.I. For each experiment, the pieces of tumor for implantation were obtained from one rat bearing a tumor transplanted approximately two weeks earlier. The donor rats were sacrificed with CO_2 , submerged in disinfectant solution, and the tumors removed aseptically. The tumors were then rinsed in a sterile balanced salt solution before being freed of connective tissue and necrotic material in small plastic dishes on ice. The remaining non-necrotic tumor tissue was cut into small fragments and implanted within 30 minutes of sacrifice.

Experiment 1 - Intact Retired Breeders. Intact retired breeders were divided into two groups and fed either the high fat diet (n=19) or the low fat diet (n=15) from four weeks prior to tumor implantation to the end of the experiment, 40 days after implantation. Total time on the diet was 10 weeks.

Experiment 2 - Ovariectomized Retired Breeders. The animals were again divided into two groups (n=13 each) and fed the various diets from four weeks prior to being ovariectomized to the end of the experiment, 40 days after implantation. For ovariectomy, the animals were anesthetized with sodium pentobarbital and the ovaries were removed through bilateral incisions in the dorsal body wall. The animals were then allowed to recover for four weeks prior to tumor implantation.

The total time on the diet was 14 weeks.

Experiment 3 - Young Virgins. Five week old female weanlings were divided into two groups (n=12 each) and fed either the high fat diet or the low fat diet. At nine weeks of age, a small piece of tumor was implanted into each rat. Diets were continued to the end of the experiment, 40 days after tumor implantation. The total time on the diet was 10 weeks.

Observation Schedule and Necropsy. In all three experiments, tumor growth was monitored twice a week using calipers to record two diameters of each tumor. Data on tumor size were expressed as the geometric mean of the tumor diameters ($\sqrt{d_1 \times d_2}$). Body weights in all three experiments were taken weekly until tumor transplantation, after which they were taken twice a week.

All rats were sacrificed with CO_2 40 days after tumor implantation and necropsies performed. Wet weights of the body and tumor, and sites and extent of metastases were recorded. Lymph nodes were judged to be enlarged if the volume was more than twice normal size.

Data Calculations and Analysis. The extent of lung metastases was determined according to the technique of Welch et al. (1983) which yields an estimate of the total tumor volume of metastatic nodules visible on the lung surface. Metastases with diameters under 1 mm are assumed to have a radius of 0.5 mm; those with diameters from 1-3 mm are assigned a radius of 1.5 mm, and those over 3mm in

diameter are measured individually and their radii figured. Individual tumor volumes are then summed to produce a value of lung tumor volume for each animal.

Analysis of the means of total tumor volume (log transformation) in the lungs of the intact and ovariectomized retired breeders on either a low fat or high fat diet was done using the Student's t test. Linear regression analysis and the two tailed t test were performed to determine any significant differences in weight gain and rate of tumor growth of the tumors between the two diet groups in each experiment. All averaged data were expressed as mean ± standard deviation.

RESULTS

Experiment 1 - Intact Retired Breeders

Following implantation, palpable primary tumors were evident within seven days. There was no significant difference in the rate of tumor growth between the different dietary groups throughout the experiment (Text-Fig.1). At sacrifice, the mean tumor diameter in the high fat group was 38.4±5.8 mm compared to 36.2±6.6 mm in the low fat group; mean tumor weights were also very similar (Table 2).

At necropsy, the lungs of 33 of 34 of the rats contained visible metastases and the extent of metastatic tumor was analyzed (Table 3). The mean volume of lung metastases in the high fat dietary group was significantly higher ($p \leq 0.05$) than the mean lung tumor volume of the low fat dietary group. In the high fat group, 84.2% of the animals had nodules that were 1 to 3 mm in diameter and 36.8% had nodules with a diameter of more than 3 mm. In the low fat group, 53.3% of the rats had 1 to 3 mm nodules and 13.3% had nodules with a diameter over 3 mm. Comparison of the median tumor volumes in the lungs of the two dietary groups also demonstrated the effect of the high fat diet (Table 3).

Body weights were recorded weekly during the 4 week pre-feeding period and for 6 weeks following tumor implantation (Table 2). Linear regression analysis of

Figure 1. Geometric mean of tumor diameter of intact or ovariectomized RBs fed different diets. Intact HF, n=19 (●); LF, n=15 (○). Ovariectomized HF, n=13 (■); LF, n=13 (□).

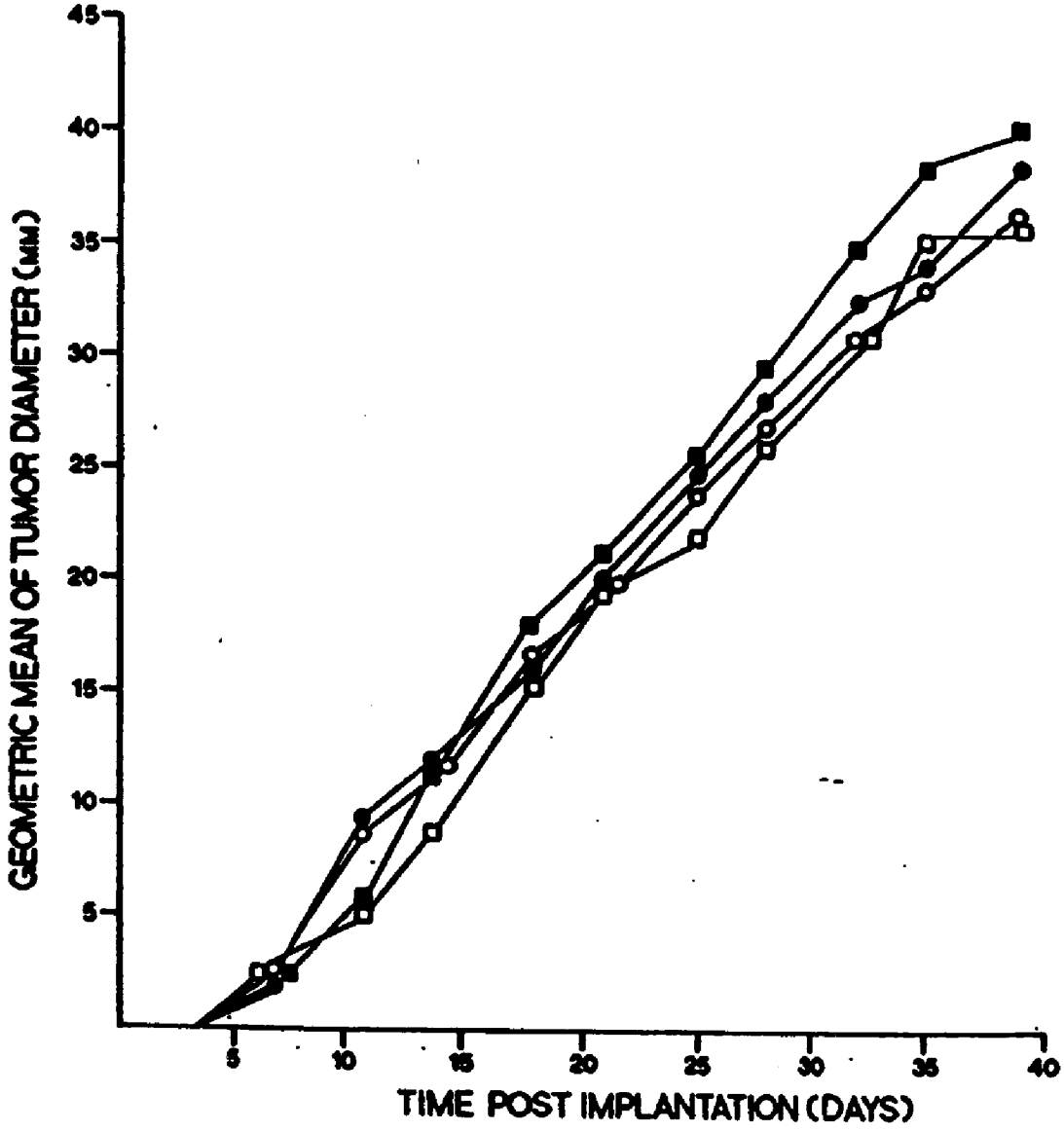

GEOMETRIC MEAN OF TUMOR DIAMETER (mm)
45
40
35
30
25
20
15
10
5
5
10
15
20
25
30
35
40
TIME POST IMPLANTATION (DAYS)

Table 2. Body Weights and Primary Tumor Weights of Rats on Diets Differing in Fat Content

Experiment	Mean Body Weight (g ± SD)			Mean Tumor Weight (g ± SD)	
	At Initiation of Diet	At Tumor Implant	At Sacrifice	At Sacrifice	As Percent of body wt
1. Intact Retired Breeders					
High Fat	231 ± 17	266 ± 25	274 ± 33	19.3 ± 7.8	7.0%
Low Fat	235 ± 14	255 ± 27	257 ± 30	18.1 ± 6.4	7.0%
2. Ovariectomized Retired Breeders					
High Fat	227 ± 12	276 ± 18	298 ± 19	26.0 ± 13.3	8.7%
Low Fat	229 ± 19	258 ± 28	280 ± 27	22.2 ± 9.4	7.9%
3. Intact Young Virgins					
High Fat	58 ± 6	130 ± 6	160 ± 15	23.5 ± 8.0	14.7%
Low Fat	57 ± 6	127 ± 15	156 ± 8	22.3 ± 8.0	14.3%

Table 3. Volume of Pulmonary Metastases in Intact Retired Breeder Females

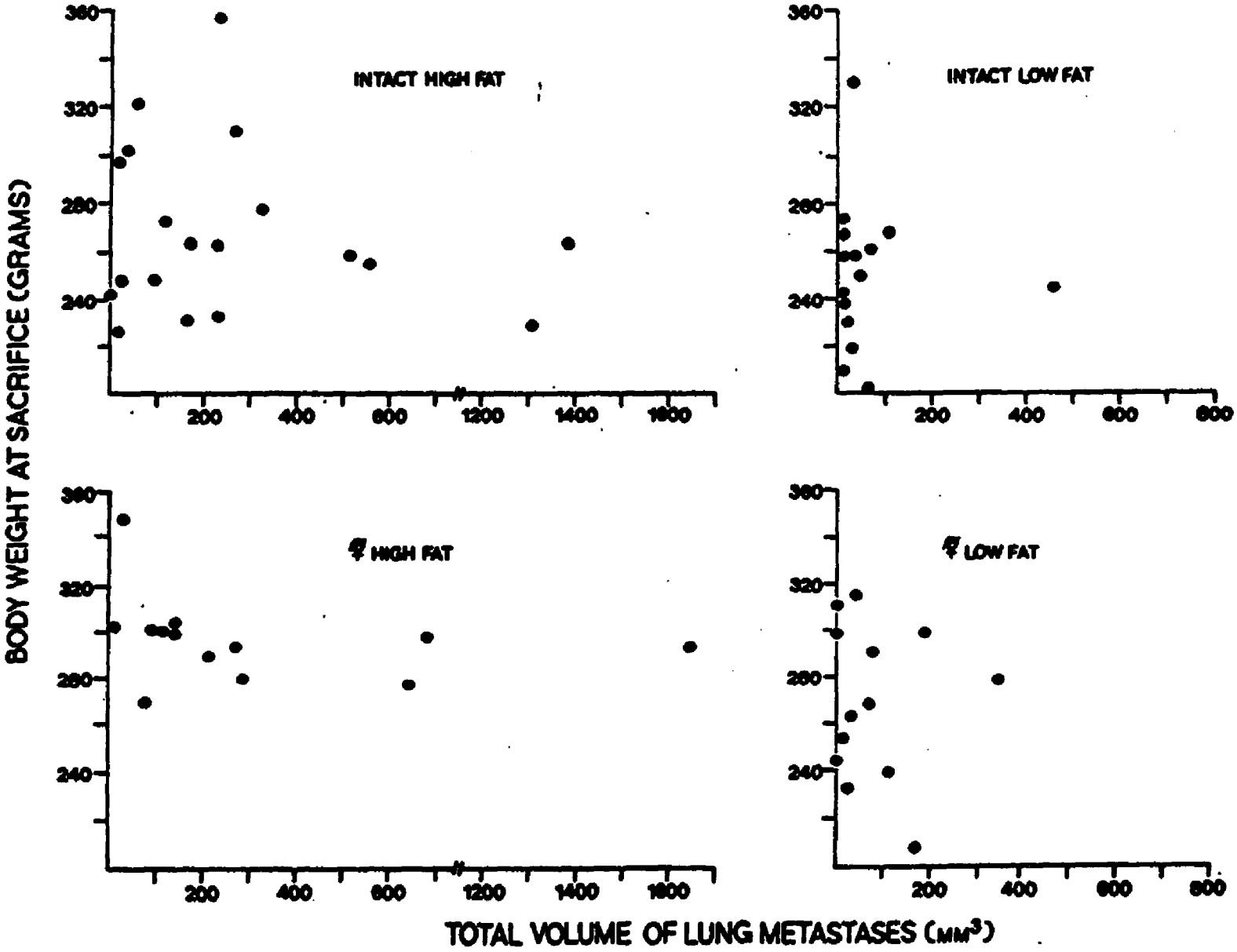
Rat Number	High Fat Diet				Low Fat Diet			
	Number of Nodules			Total Volume (mm^3)	Number of Nodules			Total Volume (mm^3)
	1mm	1-3mm	3mm		1mm	1-3mm	3mm	
1	44	96	0	1380.1	42	27	1	469.2
2	0	0	0	0.0	7	0	1	69.2
3	44	32	1	580.9	10	0	0	5.2
4	33	5	0	89.0	7	1	0	17.9
5	13	1	0	20.9	81	5	0	114.0
6	35	8	0	5.7	13	0	0	6.9
7	5	9	0	533.5	8	2	0	32.5
8	16	5	3	162.8	4	2	0	30.4
9	4	0	0	14.7	3	0	0	1.6
10	10	10	2	1308.0	7	0	0	3.7
11	56	12	0	270.4	2	0	0	1.4
12	0	10	2	25.0	11	0	0	76.5
13	1	3	1	233.5	5	0	0	2.6
14	21	20	1	327.1	0	2	0	28.3
15	7	14	1	235.0	12	2	0	34.5
16	8	6	0	117.2				
17	0	12	0	169.6				
18	1	4	1	381.5				
19	18	18	0	263.7				
				X= 318.0±396.0[a]				X = 59.6 ± 188.0
				median = 233.5				median = 28.3

[a]Significantly different from LF group, $p \leq 0.05$.

these data indicated a significant difference ($p \leq 0.05$) between diet groups despite the fact that the low fat animals actually consumed more calories than the animals in the high fat group - 38g/3 rats/day (= ~58 kcal/rat/day) in the high fat group; 52g/3rats/day (= ~63 kcal/rat/day) in the low fat group. In order to determine whether the increase in weight found in the high fat group could be responsible for the greater lung metastatic burden seen in this group, the data on total volume of lung metastases per animal were graphed as a function of body weight and analyzed (Text-Fig.2). It is apparent that there is no correlation between body weight and metastatic burden in either the high fat ($R=-.180$, $p \leq 0.05$) or the low fat ($R=-0.095$, $p \leq 0.05$) groups, indicating that the minor differences in body weight cannot account for the significant increases in the volume of pulmonary metastases seen in rats on the high fat diet.

At necropsy, other metastatic sites were observed, including the axial, inguinal and lumbar lymph nodes. In the high fat dietary group, 80% of the animals had enlarged axial lymph nodes while in the low fat dietary group, only 40% of the axial lymph nodes were enlarged. There was a single metastatic focus in the kidney of one high fat animal and one low fat animal had a single metastatic lesion in the liver.

Figure 2. Volume of lung metastases as related to body weight of each RB female at sacrifice. ♀ = ovariectomized prior to tumor implant.


INTACT HIGH FAT
INTACT LOW FAT
♀ HIGH FAT
♀ LOW FAT
BODY WEIGHT AT SACRIFICE (GRAMS)
TOTAL VOLUME OF LUNG METASTASES (mm³)
360
320
280
240
200
400
600
1200
1400
1600
800

Experiment 2 - Ovariectomized Retired Breeders

Retired breeder females placed on a high fat or low fat diet were ovariectomized 4 weeks prior to tumor implantation. Within 7 days of tumor implantation, palpable primary tumors developed. In contrast to Experiment 1, there was a significent difference in the rate of primary tumor growth between the two dietary groups (Text-Fig.1). At sacrifice, the mean tumor diameter was 40.3mm ±8.5mm in the high fat dietary group and 36.04mm ± 5.2mm in the low fat dietary group; the wet weight of the tumors in the high fat group was also higher (Table 2). It should be noted that the ovariectomized groups had been on the test diets for a longer time than the intact retired breeders used in Experiment 1, i.e. 4 weeks preceeding tumor implantation.

The metastatic spread of tumor tissue was analyzed at necropsy (Table 4). The lungs of 93% of the animals contained some visible metastases. Again, the mean volume of lung metastases in the high fat group was significantly higher than that of the low fat group ($p \leq 0.05$). Of the animals in the high fat group, 92.3% of their lungs contained nodules with a diameter of 1-3mm and 53.8% contained nodules with a diameter of more than 3 mm. In contrast, only 46.1% of the lungs of the animals in the low fat group contained 1-3 mm diameter nodules and 38.5% contained nodules with a diameter greater than 3 mm. Also,

Table 4. Volume of Pulmonary Metastases in Ovariectomized Retired Breeder Females

Rat Number	High Fat Diet				Low Fat Diet			
	Number of Nodules			Total Volume (mm^3)	Number of Nodules			Total Volume (mm^3)
	1mm	1-3mm	3mm		1mm	1-3mm	3mm	
1	10	6	0	90.1	2	0	1	66.5
2	6	3	1	80.0	4	5	0	72.8
3	47	37	2	648.6	5	17	1	347.1
4	5	1	0	20.9	1	2	0	28.8
5	12	7	1	138.8	13	2	1	112.5
6	35	8	0	137.2	9	2	0	38.3
7	5	9	0	129.8	5	9	1	195.0
8	16	5	3	353.0	0	0	0	0.0
9	4	0	0	2.9	2	0	1	65.9
10	10	10	2	1648.5	0	0	0	0.0
11	56	12	0	298.9	0	0	0	0.0
12	0	10	2	690.5	17	0	0	8.9
13	1	3	1	222.5	10	0	0	5.2
				$\bar{X} = 343.2 \pm 448.8$[a]				$\bar{X} = 72.4 \pm 99.8$
				median = 137.2				median = 38.3

[a]Significantly different from LF group, $p \leq 0.05$.

the median lung tumor volume of the high fat group was substantially higher than in the low fat group (Table 4).

As in Experiment 1, the data on body weights were analyzed by a linear regression method; a significant trend was noted ($p \leq 0.05$) with rats on the high fat diet being heavier. The data on the total voume of lung metastases in the two ovariectomized diet groups as a function of body weight is shown in Text-Fig. 2. Again, no positive relationship was found between these two parameters (low fat - $R=0.054$, p 0.05; high fat - $R=-0.298, p \leq 0.05$), indicating that the increase in body weight noted in the high fat group is not correlated with increase in pulmonary tumor burden.

Other metastatic sites included the axial and inguinal lymph nodes. In both the high fat and low fat dietary groups, 70% of the animals had enlarged axial lymph nodes. No distant organ metastases other than the lungs were observed in either experimental group.

Growth Potential of Pulmonary Metastases

To insure that what was being counted as a 1mm lung nodule was, in fact, tumor tissue, 3 representative single pulmonary nodules of 1mm diameter from both high and low fat animals were implanted into 6 week old female rats to check for primary tumor growth and metastases. Primary tumors did arise from these nodules, and at necropsy, metastatic foci with a diameter of at least 1-3 mm were

found in the lungs. Two mm^3 pieces of the primary tumor from both high fat and low fat animals were also implanted into 6 week old female rats. As with the pulmonary nodule implants, a primary tumor developed in each case and metastatic foci of 1-3 mm in diameter were found in the lungs at necropsy.

Experiment 3 - Young Virgins

The growth of the primary tumor was followed for thirty nine days. Palpable tumors appeared in most rats within 7 days. As in Experiment 1, there was no significant difference in the rate of primary tumor growth between the different dietary groups throughout the experiment. At sacrifice, the mean tumor diameter in the high fat group was 37.5 ± 4.6 mm, compared to 38.3 ± 5.6 mm in the low fat group; mean tumor weights are presented in Table 2.

At necropsy, the lungs of 100% of the rats in both diet groups contained abundant metastases (Table 5). Expressed either as a mean or a median, there was no significant difference in the total metastatic volume between diet groups. The volume of visible metastases on the lungs of young virgins in the high fat group ranged from 85.3 mm^3 to 1596.8 mm^3, in the low fat group the range was from 69.1 mm^3 to 2054.9 mm^3.

Weight gain in the young virgins was rapid and there

Table 5. Volume of Pulmonary Metastases in Young Virgin Females

Rat Number	High Fat Diet				Low Fat Diet			
	Number of Nodules			Total Volume (mm^3)	Number of Nodules			Total Volume (mm^3)
	1mm	1-3mm	3mm		1mm	1-3mm	3mm	
1	106	39	2	1596.8	110	86	3	1373.9
2	139	71	4	1242.4	111	38	1	768.4
3	161	3	0	126.7	139	6	0	156.6
4	138	41	2	748.9	60	45	1	810.4
5	35	6	1	147.1	90	11	2	504.2
6	98	48	0	728.8	197	64	2	2054.9
7	10	14	5	370.6	79	27	2	804.3
8	102	10	1	222.2	105	1	0	69.1
9	82	3	0	85.3	135	34	3	683.8
10	43	29	1	466.0	57	18	2	906.8
11	108	23	4	754.4	49	2	2	152.9
12	73	33	4	952.9	68	6	2	189.4
				X= 620.0 ± 475[a]				X= 706.4 ± 576.3
				median = 597.4				median = 726.0

[a] Not significantly different from LF group, $p \leq 0.05$

was no significant difference in average body weights after the first four weeks on the diets . At sacrifice the mean body weight in the high fat group was very similar to that in the low fat group (Table 2).

DISCUSSION

These results on aged female rats fed a high or low fat diet both prior to and following tumor implantation represent the first report in a laboratory animal model demonstrating that modulation of mammary tumor metastasis to distant organs is possible by altering the proportion of fat in the diet. Comparable dietary regimens in young virgin rats were ineffective in modulating the growth of metastases in the lungs, illustrating the importance of age of the host (and reproductive history) in developing realistic animal models for human breast cancer.

In both intact and ovariectomized hosts, the high fat retired breeders had significantly more pulmonary tumor volume than the low fat animals. Primary tumor growth in intact retired breeders was almost identical between the dietary groups. While the high fat rats tended to gain more weight, this weight difference does not appear to be the basis for the increased metastatic tumor burden in the high fat rats since there was no correlation between metastatic burden and body weight either at the time of implant or at sacrifice. Similar results were obtained in Experiment 2 using ovariectomized retired breeder animals, suggesting a stimulatory role of the high fat diet. In order to confirm a positive role of a high fat diet in the growth of pulmonary metastases in the absence of body weight differences, we intend to repeat these experiments using a

pair-fed design, instead of ad libitum food access (Thompson et al. 1985).

In Experiment 3, there were no observable differences in either the primary tumor growth or weight gain between the different dietary groups of the YV animals. Unlike the results of the first two experiments, however, there was no significant difference in average metastatic tumor volume in the lungs of the animals in the different dietary groups. The lungs of both dietary groups contained abundant metastases, comparable to data presented in our earlier report (Boylan & Cohen 1986).

When considering the difference in response in the 2 age groups, one should note that the final average diameters of the primary tumor and the average tumor weights were very similar for both age groups. However, the average body weights of the YV's and intact RB animals differed by over 100 g. When the average tumor weights were expressed as a proportion of the final body weights, tumor weight accounted for 14-15% of the total body weight of the YV's, while only 7-9% of the final body weight of the older animals was derived from the tumor. At the time of sacrifice, the young animals in both dietary groups had evidence of cachexia, as indicated by their scruffy pelt appearance and decrease in body weight. At necropsy, the amount of subcutaneous fat was negligible and the abdominal fat deposits were almost undetectable. In contrast, at a comparable time after tumor implantation,

tumor growth appeared to have substantially less systemic effect on the older hosts whose general appearance continued to be normal. In addition, at necropsy, the TB's had retained most of their subcutaneous fat and a considerable amount of internal fat deposits. Finally, in a comparison of the average volume of metastatic nodules in the lungs, the young animals in the HF group had almost twice as much tumor volume as the older animals in the HF group, while the younger animals in the LF group had over ten times the metastatic burden of the LF older animals. These results suggest that the pulmonary metastases from the 13762 mammary tumor grew more rapidly in the younger animals. Because of this rapid progress, there may not have been enough time for the different diets to exert their influence in the YVs. In contrast, possibly because of the slower progression of neoplasm in the older animals, a beneficial influence of the LF diet on the extent of metastases to the lungs was able to be observed.

The influence of dietary fat on the growth of pulmonary metastases could be operating through one (or more) of the following mechanisms: 1) perturbation of the hormonal milieu, 2) alterations in the tumor cell membranes, and 3) modulation of the immune function (Cohen 1986; Welsch et al. 1983)

We consider the first alternative unlikely, since the effect of the different diets was comparable whether the RBs were intact or ovariectomized. Furthermore,

cytosolic estrogen and progesterone receptors are low or absent in the 13762 tumor grown either in the young intact or the ovariectomized host (Boylan ES: Unpublished data). And Neri et al. (1982), when comparing young intact versus ovariectomized rats, found no difference in the metastasis from the 13762 tumor.

The structure and function of the tumor cell membrane are known to be influenced by the level of dietary fat. For example, the fatty acid composition of the R3230Ac mammary tumor membrane was altered significantly, depending on whether the rats were fed a 0.5% corn oil or a 20% cottnseed oil diet (Feldman & Hilf 1985). Such effects could have dramatic consequences on energy metabolism and growth.

Since variation in the quantity and type of dietary fat is recognized to affect the function of many components of the immune system, our data could also be explained through a suppressive effect of the high polyunsaturated fat diet on one or more of the cells of the immune system. Johnston (1985) has summarized much of the recent literature on dietary fat and immunity in various rodent models and has concluded that high polyunsaturated diets generally depress the mitogenic response of lymphocytes, but the source of the lymphocyte is and important determinant of response. Thomas and Erickson (1985) have shown that increasing the amount of polyunsaturated fat in the diet decreased the ability of

cytotoxic T-lymphocytes from the BALB/cAnN mouse to lyse mammary tumor cells (line 168) in vitro.

The diet-related effects on tumor cell membranes and immune cell function could also be indirect, operationg through diet-induced alterations of prostaglandin production. Fulton and Heppner (1985) demonstrated a positive relationship between high levels of prostaglandin E and metastatic potential in several mouse mammary tumor lines; furthermore, metastases from two highly metastatic lines were inhibited by the prostaglandin inhibitor indomethacin. In contrast, Fulton et al. (1985) reported that levels of prostaglandin E_2 and prostaglandin F_2 in tumor samples from stage I and II breast cancer patients failed to correlate with the degree of lymph node involvement or with the likelihood of developing recurrent disease or death. Johnston (1985) has reviewed the literature linking dietary fat and its effect on eicosanoid production. While there are many demonstrable dietary effects on eicosanoid production, there appears to be no clear mechanism by which the altered levels of prostaglandins affect the functioning of the immune system. While we cannot evaluate the various alternate mechanisms discussed, we now have a model system whereby the biochemical basis of the dietary effects on metastasis can be explored. Furthermore, these data lend direct experimental support for the clinical dietary interventions now undergoing feasibility trials in breast cancer patients (National Institutes of Health 1986).

Chapter 2

EFFECTS OF RECIPROCAL CHANGES OF DIETS DIFFERING IN FAT CONTENT ON PULMONARY METASTASIS FROM THE 13762 RAT MAMMARY TUMOR

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ABSTRACT

The effect of changing the amount of polyunsaturated fat in the diet of aged female Fischer 344 rats at the time of tumor implant on metastasis from the 13762 transplantable mammary tumor was studied. Three experiments were performed: 1) Retired breeders (RB), maintained on standard commercial chows until 10-12 mo of age, were transferred to high fat (HF, 23% corn oil) or low fat (LF, 5% corn oil) diets for 4 weeks; at tumor implant, half of each group were kept on their original diets , while half were switched to the other diet (i.e., HF→HF, HF→LF, LF→LF, LF→HF); 2) Aged virgins, 14-16 mo old, were fed HF and LF diets from weaning; at tumor implant, the LF group stayed on the LF diet, while half the HF group remained on the HF diet and half were changed to LF; 3) RB were fed Purina Rodent Chow (RC, 5% mainly saturated fat) until tumor implant when they were placed on either the HF or LF diets. Six weeeks after tumor implant, all rats were necropsied, and the extent of pulmonary metastasis determined. Data were expressed as volume of pulmonary metastases. In experiment 1, animals maintained on a HF diet or switched to a HF diet at implant had significantly more pulmonary metastases than those animals kept on a LF or changed to a LF diet. Likewise in experiment 2, pulmonary metastasis was less in rats which were fed a HF diet from weaning, then switched to LF at tumor implant

than in the animals maintained on a HF diet both before and after tumor implant. Finally, in experiment 3, when rats were switched from RC to either the HF or LF diet at tumor implant, there was no significant difference in the extent of pulmonary metastasis between the two groups; in both, the extent of metastasis was comparable to that seen in animals maintained on the LF corn oil diet. Data on metastasis was also examined in light of body weight, growth of the primary tumor and food disappearance. These results suggest that the amount of fat consumed by aged rats after tumor implant is an important determinant on the extent of pulmonary metastasis from the 13762 mammary tumor. However, a period of pre-feeding the semi-purified diets appears to be required in order for the HF corn oil diet to stimulate metastasis in this system.

INTRODUCTION

The breast cancer death rate in the United States has not changed appreciably in the last 20 years even though methods for early detection and therapy have improved (Cancer Facts and Figures--1987). There is an obvious need for more effective treatments and adjuvant therapies for metastatic disease. Dietary intervention is one such adjuvant therapy which has been considered (Clifford et al. 1986), based on several epidemiological studies which indicate that the quantity and quality of dietary fat can affect the initiation and growth of breast cancer in humans (Miller 1986; Gregorio et al. 1985; Sakamoto et al. 1979). Other studies, however, have not been able to demonstrate an association between reduced fat intake and a lower risk for breast cancer or improved survival (LeMarchand et al. 1985). Serious consideration has been given to implementation of large scale clinical trials where breast cancer patients and women with no evidence of breast cancer would be stratified into a "normal" diet group and an intervention group, counseled to follow a diet where only 20% of the calories are derived from fat (Clifford et al. 1986). In early 1988, the National Cancer Institute decided not to go forward with the protocol in its existing form, reportedly for reasons of experimental design, problems regarding sample size and extremely high cost. Thus, at the present time, there are no clinical trials on

the effect of lowering fat consumption on breast cancer risk or as adjuvant therapy.

In contrast to the data on humans, studies in animal models using chemically-induced and transplantable mammary adenocarcinomas have been more consistent in showing that diets containing a high percent of calories as fat can affect the initiation of mammary tumors[3] and subsequent growth of these tumors (Cohen 1986; Rogers et al. 1981; Carroll & Khor 1983). In addition to the numerous reports relating dietary fat quality and quantity to mammary tumor initiation and promotion, modulation of the amount of time the diets were fed has also been studied. Kritchevsky et al. (1984) looked at the effect of feeding a HF coconut oil diet (a saturated fat of medium length triglycerides) before and/or after administration of DMBA. They found that female Sprague-Dawley rats given the HF diet before administration of DMBA, and then kept on a HF diet or switched to an isocaloric LF (5% fat) diet, had twice as many palpable tumors as rats on the LF diet before being given DMBA, then kept on a LF diet or switched to the HF diet. These authors suggest that a high fat coconut oil diet fed before DMBA administration, but not after, affected the initiation of the MT's. Dao and Chan (1983) studied the role of a PUFA HF (corn oil) diet on NMU-induced MT's. Their results indicated that the amount of time a PUFA HF diet is fed, before and after carcinogen administration, correlated positively with the incidence

and growth of NMU-induced MT's. If rats were pre-fed the HF diet four weeks prior to or after being given NMU, there was no effect on MT incidence. In contrast, if the feeding period was 6 weeks or more, tumor incidence increased proportionately.

The above experiments were concerned with the effect of the type of dietary fat and length of feeding time on the incidence and growth of chemically induced mammary adenocarcinomas. However, the problem of metastasis from primary MT's had not been investigated, since these chemically-induced tumors rarely (DMBA) or infrequently (NMU) metastasize. Using the 13762 transplantable MT which metastasizes reproducibly to the lungs and regional lymph nodes, we have demonstrated that a PUFA HF diet resulted in a significant stimulation in pulmonary metastasis in retired breeder female rats (Katz & Boylan 1987a). With these studies in mind, we performed experiments which addressed whether the amount of time a PUFA HF diet was fed before and/or after implantation of the 13762 MT affected the extent of pulmonary metastasis. Animals were either started on the diet at the time of tumor implant or pre-fed the HF or LF diet for four weeks or from weaning. Other groups pre-fed the HF or LF diets were switched to the opposite diet at the time of implant. Our experiments suggest that the type of diet fed before and after implantation of the 13762 MT affects the extent of pulmonary metastases seen in the animals. A preliminary

report of some of these data was presented at the 1987 meeting of the American Association for Cancer Research (Katz & Boylan 1987b).

MATERIALS AND METHODS

Animals and Diets

Female Fischer 344 retired breeder rats, age 10-12 months, were purchased from Charles River Breeding Laboratories, Wilmington, MA for use in experiments 1 and 3 (see Figure 1). For experiment 2 (Figure 1), virgin female Fischer 344 rats raised in our animal facility were used at age 12-16 months. Animals were routinely housed 3 to a cage in a temperature- and light controlled room, and were fed one of three diets: 1) Purina Rodent Chow 5001, Ralston Purina Co., St. Louis, MO, which has a fat content of 6%, mainly from bleachable animal fat: beef tallow (30%), and pork lard (70%); 2) High Fat semi-purified rat diet (23% corn oil); 3) Low Fat semi-purified rat diet (5% corn oil). The semi-purified diets (Table 1) were obtained from Bio-Serv Inc., Frenchtown, NJ, in pellet form, stored in the dark at 4°C, and used within 4 weeks. The comparative fatty acid profiles of the three diets are presented in Table 2. The diets were designed to be isocaloric but were not balanced nutritionally. Micronutrients in both diets exceeded minimum nutritional requirements (Bieri et al. 1977). The diets were fed for varying periods of time as shown in Figure 1. The 3 experiments were done consecutively; 2 separate trials were combined in experiment 3. Group sizes for each experiment are noted in Figure 2 and Table 3.

Figure 1. Dietary protocols for Experiments 1, 2, and 3, showing timing of diet transitions relative to time of tumor implantation. RC diet is Purina rat chow. HF and LF diets are semi-purified formulations obtained from Bio-Serv, Inc., containing either 23% or 5% fat from corn oil. Group sizes are noted in Fig. 2 where each dot represents a rat.

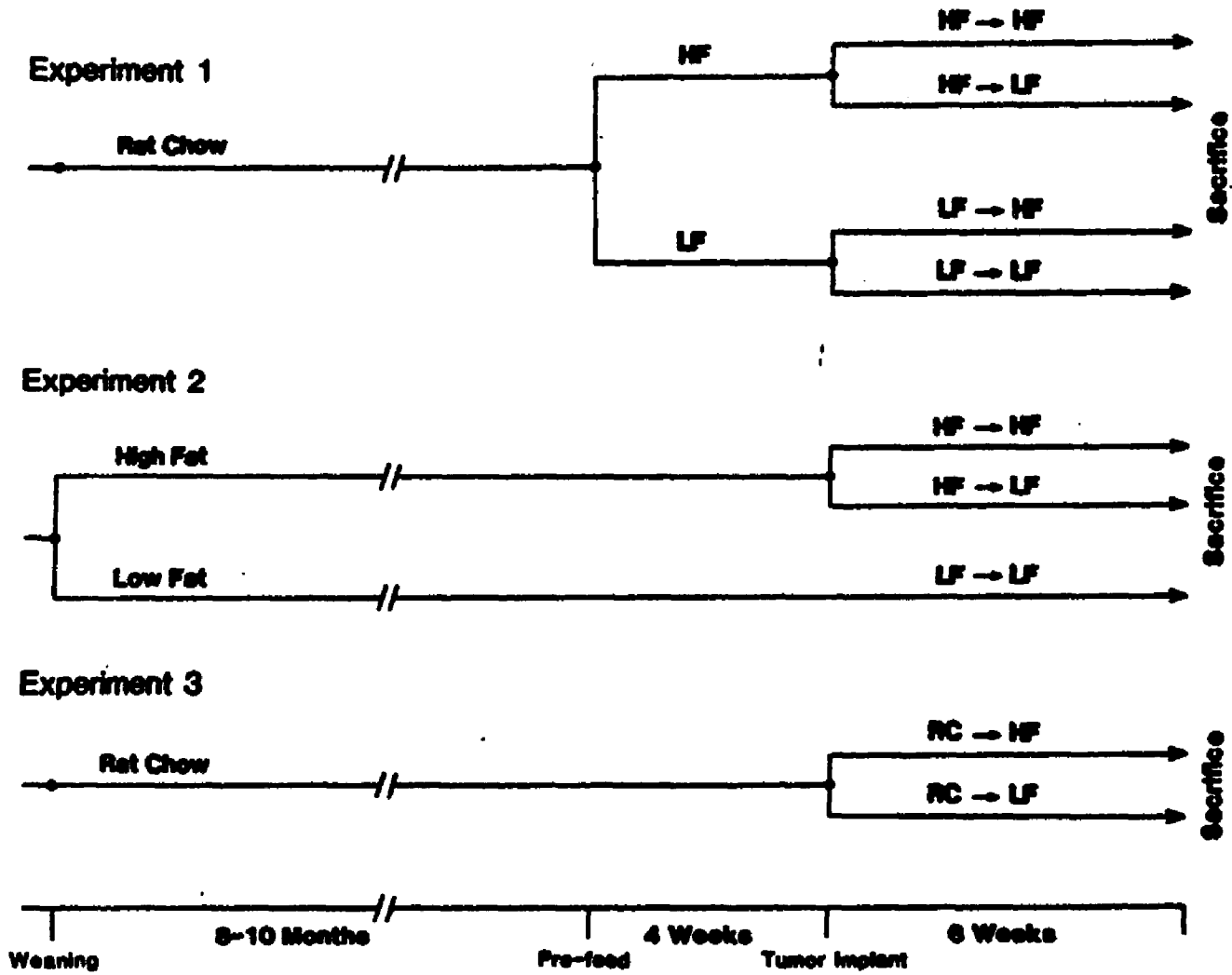

Experiment 1
Rat Chow
HF
LF
HF → HF
HF → LF
LF → HF
LF → LF
Sacrifice
Experiment 2
High Fat
Low Fat
HF → HF
HF → LF
LF → LF
Sacrifice
Experiment 3
Rat Chow
RC → HF
RC → LF
Sacrifice
Weaning
8-10 Months
Pre-feed
4 Weeks
Tumor Implant
6 Weeks

Table 1. Composition of Semi-Purified Low Fat and High Fat Corn Oil Diets

	Composition (%)	
	Low Fat Diet	High Fat Diet
Ingredients		
Dextrose and Maltose Dextrin	35.20	16.90
Corn Starch	23.80	23.80
Casein	23.90	23.90
Corn Oil	5.00	23.00
Alphacel	5.90	5.90
Mineral Mix[a]	4.13	4.13
Vitamin Mix[a]	1.77	1.77
DL Methionine	.30	.30
Choline Bitartrate	.24	.23
Energy Value	3.68 kcal/g	4.59 kcal/g

[a] AIN-76 Formula

Table 2. Fatty Acid Profiles of Purina Rodent Chow (5001) and Semi-purified Corn Oil Diets

	% Total Fat[a]	
Fatty Acid	Rodent Chow	Corn Oil
Linoleic, 18:2n-6	27.3	58.7
Linolenic, 18:3n-3	3.1	0.8
Oleic, 18:1n-9	30.5	26.6
Palmitic, 16:0	20.5	11.5
Stearic, 18:0	9.3	2.2
Eicosanoic, 20:5	1.3	0.2
Other	8.0	0.0

[a] Information was obtained from the suppliers, i.e., Bio-Serv, Inc., Frenchtown, NJ, for the semi-purified diets and from Purina Mills, Richmond, IN, for Purina Rodent Chow.

Figure 2. Dots represent the total volume of pulmonary metastases in individual rats after 6 wk of tumor growth in Experiments 1(A), 2(B), and 3(C). Refer to Fig. 1 for explanation of dietary protocols. LF, low fat (5% corn oil); HF, high fat (23% corn oil); RC, Purina rodent chow. Arrows denote median value in each group

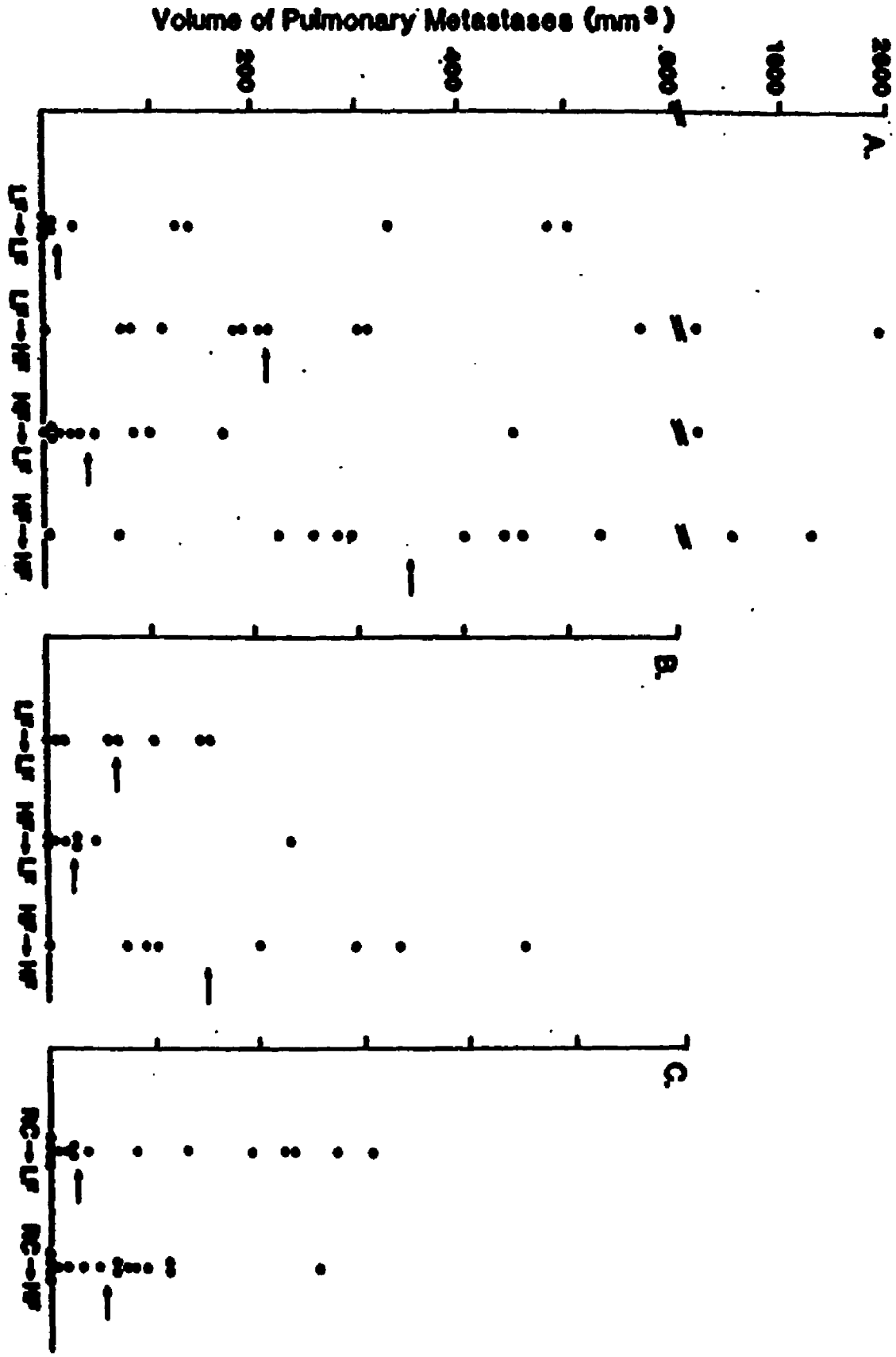

Volume of Pulmonary Metastases (mm³)
A
B
C

Table 3. Necropsy Data after 6 Weeks of Tumor Growth[a]

	Pulmonary Metastatic Volume (mm^3)		Final PrimaryTumor Weight	Correlation
	Mean ± SEM	Median		
Experiment 1				
HF→HF (n = 12)[a]	495.1 ± 149.4[b]	349.2	34.0 ± 9.0	+ 0.4367 p= 0.16
LF→LF (n = 12)	135.3 ± 58.2	28.8	22.6 ± 8.6	+ 0.6393[c] p= 0.03
HF→LF (n = 12)	149.8 ± 75.5	40.1	32.8 ± 15.0	+ 0.6322[c] p= 0.03
LF→HF (n = 13)	431.5 ± 186.0	209.4	30.0 ± 9.0	+ 0.1976 p= 0.52
Experiment 2				
HF→HF (n = 8)	192.9 ± 55.3	150.7[d]	20.0 ± 9.0	+ 0.6164 p= 0.10
LF→LF (n = 8)	67.1 ± 21.9	63.6	11.7 ± 7.2[e]	+ 0.4966 p= 0.21
HF→LF (n = 8)	45.2 ± 27.3	22.3	17.0 ± 4.3	+ 0.1713 p= 0.69
Experiment 3				
RC→HF (n = 16)	60.7 ± 16.7	56.4[f]	13.0 ± 9.0[f]	+ 0.5264 p= 0.22
RC→LF (n = 16)	95.5 ± 29.5	27.7	16.0 ± 9.0	+ 0.4916 p= 0.18

[a] Numbers in parentheses, number of rats per group.
[b] HF→HF and LF→HF groups combined significantly different from HF→LF and LF→LF groups, $p \leq 0.05$.
[c] Significant positive correlations.
[d] HF→HF group significantly greater than LF→LF and HF→LF combined, $p \leq 0.05$.
[e] LF→LF significantly different only gram the HF→HF group, $p \leq 0.05$.
[f] Not significantly different.

Tumor Implantation

Pieces ($2mm^3$) of the transplantable 13762 mammary adenocarcinoma were implanted sc into each rat just posterior to the fourth nipple. The tumor line was obtained through Dr. A. Bogden, Mason Research Institute, Worcester, MA, from the Animal and Human Tumor Bank supported by the Breast Cancer Program, National Cancer Institute, Bethesda, MD. The 13762 MT, a relatively undifferentiated adenocarcinoma, is an ovarian-independent tumor, growing well in intact and ovariectomized females (Katz & Boylan 1987a) and in intact young males (unpublished data). Cytosolic estrogen and progesterone receptors are undetectable in the tumor line (unpublished data). For each trial, the pieces of tumor for implantation were obtained from 1 rat bearing a tumor transplanted approximately 2 weeks earlier. The donor rats were sacrificed with CO_2 and submerged in disinfectant solution; the tumors were removed aseptically. The tumors were then rinsed in a sterile balanced salt solution in small plastic dishes on ice before being freed of connective tissue and necrotic material . The remaining non-necrotic tumor tissue was cut into small fragments and implanted within 30 minutes of sacrifice.

Observation Schedule and Necropsy

In all three experiments, tumor growth was monitored twice a week with calipers to record two diameters of each

tumor. Data on tumor size were expressed as the geometric mean of the tumor diameters ($\sqrt{d \times d}$). Body weights in all three experiments were taken twice a week.

All rats were sacrificed with CO_2 40 days after tumor implantation, and necropsies were performed. Wet weights of the body and tumor, and sites and extent of metastases were recorded.

Tumor Histology

Representative tumors and tumor containing lungs from rats fed HF and LF diets in each of the 3 experiments were fixed in formalin, processed through ethylene glycol monoethyl ether and toluene and embedded in paraffin. Sections were stained with hematoxylin and eosin and examined under a Nikon Optiphot microscope.

Data Calculations and Analysis

The extent of lung metastases was determined according to the technique of Welch et al. (1983), which yields an estimate of the total tumor volume of metastatic nodules visible on the lung surface. Briefly, metastases with diameters under 1 mm are assumed to have a radius of 0.5 mm; those with diameters from 1 to 3 mm are assigned a radius of 1.5 mm, and those over 3 mm in diameter are measured individually and their radii figured. Individual tumor volumes are then summed to produce a value of lung tumor volume for each animal.

Analysis of the total metastatic tumor volume data in each experiment was done using the Kruskal-Wallis and Mann-Whitney tests ; correlations were done using the Pearson test; tumor growth was analyzed using general linear models. Body weights were analyzed using Student's t test. All tests were done using programs in SAS (1986).

RESULTS

Dietary Effects of Tumor Growth and Metastasis

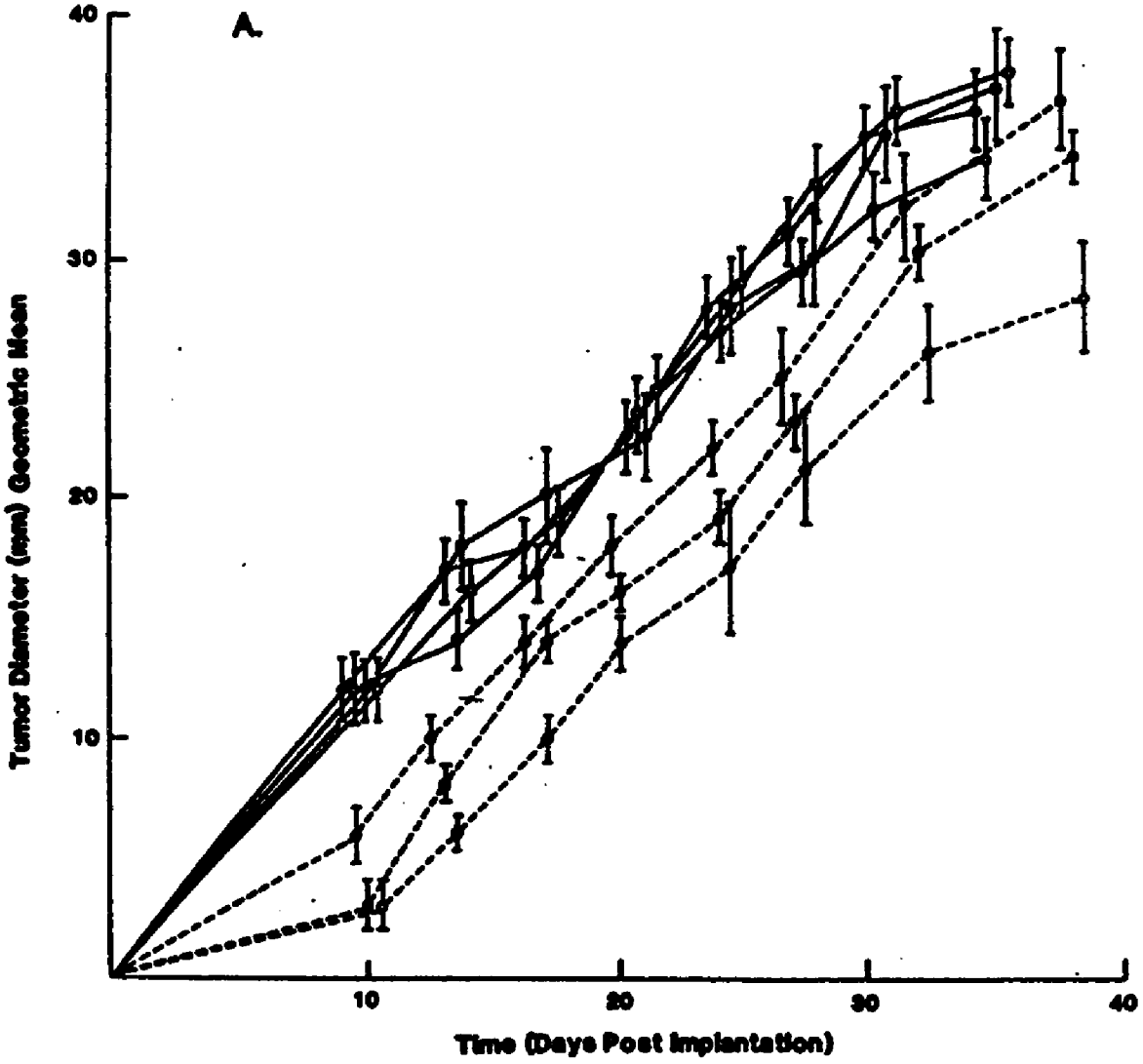
In order to determine whether the previously reported stimulatory effect of a HF corn oil diet on pulmonary metastasis (Katz & Boylan 1987a) could be modified by switching the diet at the time of tumor implant, various dietary protocols were devised. The first (Figure 1, experiment 1) involved pre-feeding retired breeders for 4 weeks on HF or LF corn oil diets; at implant, half of each group was continued on the same HF or LF diet, while the other half was transferred to the opposite diet for 6 weeks until sacrifice. Figure 2 presents the total volume of pulmonary metastases in each rat, with the median value for each dietary group noted. Table 3 summarizes the pulmonary metastasis data showing both the mean $\pm$ SEM and the median for each group. Confirming the results of our previous experiments (Katz & Boylan 1987a), the metastatic tumor burden in the HF$\rightarrow$HF group was significantly larger than that in the LF LF group ($p \leq 0.05$). Furthermore, when the 2 groups fed the HF diet between tumor implant and sacrifice (HF$\rightarrow$HF and LF$\rightarrow$HF) were contrasted to the 2 groups on the LF diet for the last 6 weeks (LF$\rightarrow$LF and HF$\rightarrow$LF), the lung tumor burden was significantly greater in the former ($p \leq 0.05$). The nature of the diet during the pre-feed time period appeared to have no effect on the

extent of metastasis.

In terms of the dietary effects on the growth of the primary tumor, all 4 groups displayed essentially the same rate of primary tumor growth (Figure 3A). Data on the final tumor wet weight showed no statistically significant difference among 3 groups (HF → HF, HF → LF, LF → HF); the LF → LF group had somewhat smaller sc tumors than the others (Table 3). While there was no correlation between primary tumor wet weight and metastatic burden within either the HF → HF and LF → HF groups, there was a significant positive correlation between sc. tumor wet weight and metastases within each of the other 2 groups (Table 3). However, it should be noted that the HF → LF group, with an average sc. tumor weight of 32.4 $\pm$ 15.0 g had a metastasis level comparable to the LF LF group whose average sc. tumor weight was 22.6 $\pm$ 8.6 g. Thus, sc. tumor weight at necropsy cannot be viewed as the sole factor determining metastasis.

The second dietary regimen used aged virgin rats which had been fed HF or LF corn oil diets from weaning (Figure 1, experiment 2). Half of the HF group was kept on HF for the 6 weeks after tumor implant; half was transferred to LF. The LF group remained on LF until sacrifice. Again, the average pulmonary metastatic volume in the HF → HF group was significantly greater than that in the HF → LF group (Table 3; Figure 2, $p \leq 0.05$). The group maintained on the LF corn oil diet throughout the lifetime of the

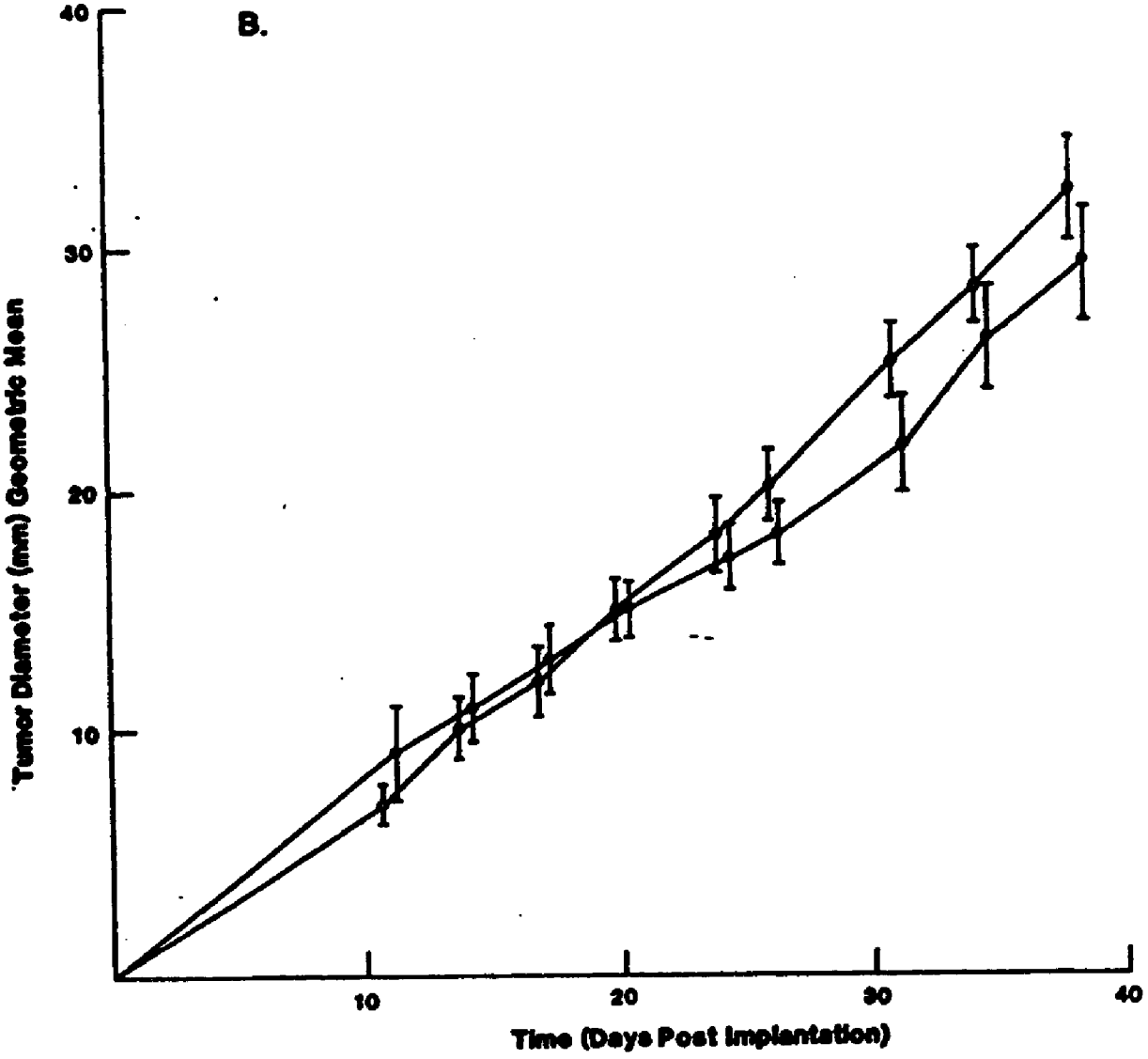
Figure 3A. Geometric mean of tumor diameter of retired breeders and aged virgins on different dietary protocols shown in Fig. 1. Experiment 1 (——): HF→HF, n=12 (●—●); LF→LF, n=12 (○—○); HF→LF, n=12 (■—■); LF→HF, n=13 (□—□). Experiment 2 (– – –): HF→HF, n=8 (●– –●); HF→LF, n=8 (■– –■); LF→LF, n=8 (○– –○). Points, mean; bars, SEM.


A.
40
30
20
10
Tumor Diameter (mm) Geometric Mean
10
20
30
40
Time (Days Post Implantation)

animals displayed a pulmonary tumor burden comparable to the HF LF group (Table 3; Figure 2). When the volumes of pulmonary metastases in groups fed the corn oil diets from weaning (experiment 2) were compared to the corresponding groups which had been raised on RC, then pre-fed the semi-purified corn oil diets before and after tumor implant (experiment 1), there was consistently less pulmonary tumor burden in those in experiment 2 (Figure 2, panel B vs. panel A). Growth of the primary tumor varied significantly among groups in experiment 2; tumors in the HF⟶HF group were larger than those in the LF⟶LF group; the HF⟶LF tumors were intermediate in size and did not vary significantly from either other group (Figure 3A). With respect to the size of the primary tumor at the end of the experiment, tumors in the LF⟶LF group weighed significantly less than those in the HF⟶HF group (Table 3). There was no significant correlation between primary tumor wet weight and pulmonary metastatic volume in any of the groups (Table 3). Primary tumor growth (Figure 3) and final primary tumor wet weight (Table 3) comparisons between experiments 1 and 2 show that groups pre-fed the corn oil diets from weaning were characterized by a slower pattern of primary tumor growth.

The third experiment involved rats which had been transferred from standard RC to HF or LF corn oil diets at the time of tumor implant (Figure 1, experiment 3). Despite having been on the HF or LF diet for the entire 6

Figure 3B. Geometric mean of tumor diameter of retired breeders on RC before and HF or LF after tumor implantation, Experiment 3 (see Fig. 1). RC HF, n=16 (●—●); RC LF, n=16 (○—○). Points, mean; bars, SEM.


B.
40
30
20
10
Tumor Diameter (mm) Geometric Mean
10
20
30
40
Time (Days Post Implantation)

weeks between tumor implant and necropsy, there was no significant difference in the average pulmonary metastatic tumor volume between the 2 groups (Figure 2, panel C; Table 3, $p \leq 0.05$). The pulmonary tumor burdens found in both of these groups (RC→HF and RC→LF) were much lower than those found in any of the previously mentioned groups which had been fed the HF corn oil diet between tumor implant and sacrifice, and comparable to the pulmonary tumor burdens found in rats maintained for either short or long periods on a LF corn oil diet (cf. Figure 2, panels B and C). Thus, the RC diet fed prior to implant appeared to counteract the stimulatory effect of the HF corn oil diet on pulmonary metastasis.

The RC diet fed prior to tumor implant also appeared to have a persistent effect on tumor growth. As seen in Figure 3B, primary tumor diameters of both the RC HF and RC→LF groups taken throughout the experiment were substantially lower than in the groups in experiment 1 fed corn oil diets before and after tumor implant, and were not significantly different from each other. This size difference held true for each of the two trials comprising experiment 3, indicating that differential viability of tumor inocula is not likely to be the major factor controlling the size disparity. At the time of sacrifice, the wet weights of the primary tumors did not differ significantly between the 2 diet groups (Table 3). The wet weights of the primary tumors in both groups in this

experiment were substantially less than that of all groups in experiment 1 where the average tumor wet weight at necropsy was 29.0 ± 10.7 g. Thus, the RC diet appeared to have a sustained effect on tumor growth as it did on the extent of pulmonary metastasis. There was no correlation between the pulmonary tumor burden and primary tumor wet weight in either the RC→HF group or the RC→LF group (Table 3).

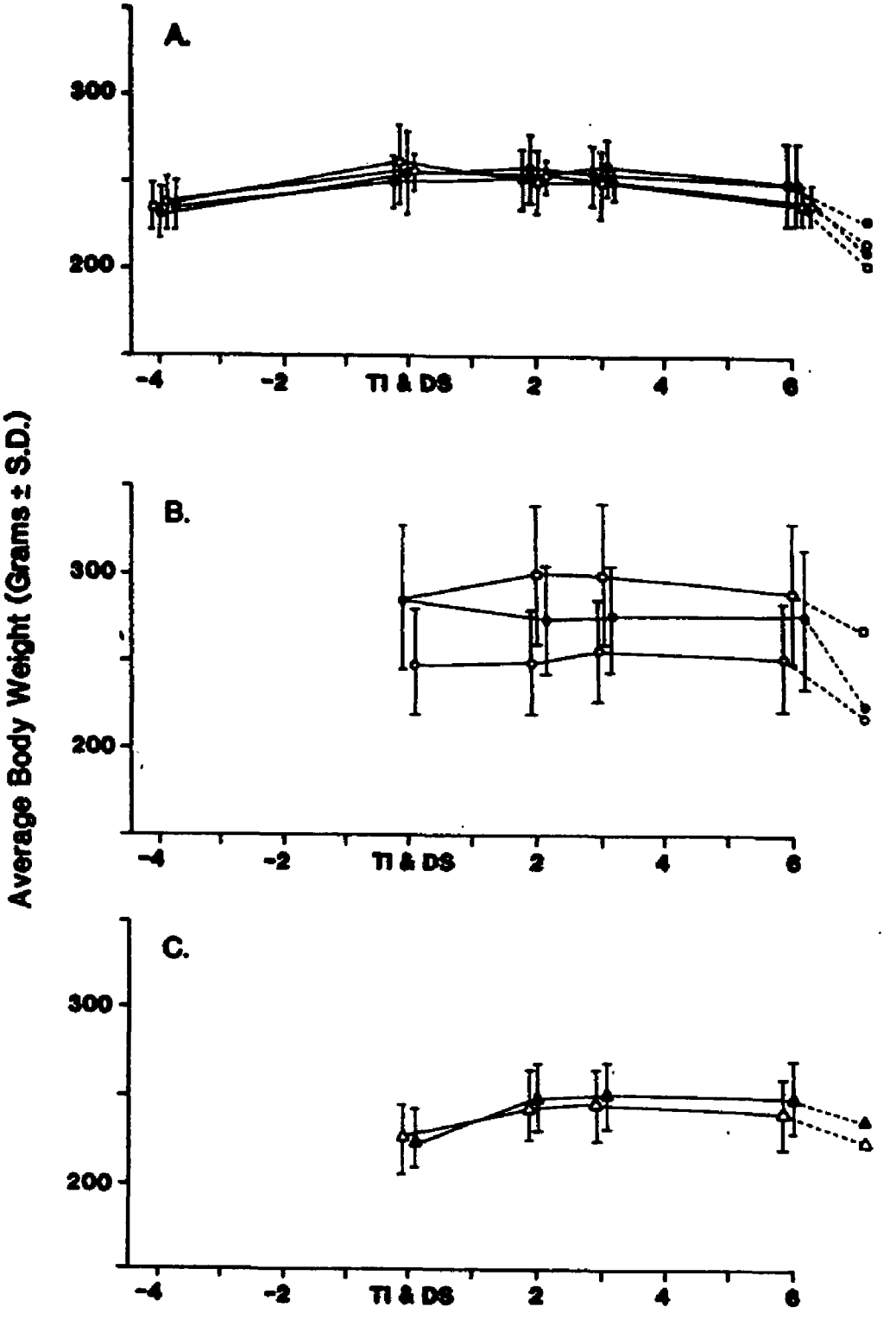
Dietary Effects on Body Weight and Food Intake

So that the effects of the different diets on pulmonary metastasis from the 13762 mammary tumor could be correlated with body weight and weight changes, rats were weighed at regular intervals throughout the experiment. In addition, the amount of food disappearance was determined at selected points before and after the diet was changed.

All 4 groups in experiment 1 (Figure 1) gained weight slightly during the 4 weeks between diet switch and tumor implantation; the amount gained did not differ significantly among the 4 groups (Figure 4, panel A). Body weights remained relatively stable in all groups throughout the next 6 weeks as the primary tumors were growing actively. Even when the body weights at sacrifice were corrected for tumor weight, it was clear that the 4 groups did not differ significantly (Figure 4A).

When weight gain during the 4 week pre-feed period

Figure 4. Average body weights for rats in Experiments 1(A), 2(B), and 3(C) over time. Time shown as before and after date of tumor implantation and dietary switch (TI & DS); refer to Fig. 1 for explanation on dietary protocols. In A and B, ● = HF→HF; ■ = LF→HF; □ = HF→LF; ○ = LF→LF. In C, ▲ = RC→HF; △ = RC→LF. Last points on X axis represent body weights at sacrifice (6wk post implant) with wet weights of primary tumor deducted.


A.
B.
C.
300
200
-4
-2
TI & DS
2
4
6
Average Body Weight (Grams ± S.D.)
Time (Weeks) Pre/Post Tumor Implantation & Diet Switch

was compared to the extent of pulmonary metastasis, the only group to show any significant correlation was the HF HF group, where rats gaining more weight tended to have more lung tumor burden ($r=0.6512$, $p \leq 0.02$).

Figure 4, panel B, presents the data on body weights for groups in experiment 2. At the time of tumor implant, rats which had been on the HF diet from weaning weighed significantly more than the LF-fed group. The body weights of these rats at tumor implant were roughly comparable to those in experiment 1 after 4 weeks on the semi-purified corn oil diets (cf. experiment 2 vs. 1, Figure 4). Despite a switch from HF to LF, body weights of the rats were maintained. At sacrifice, body weights with tumor also failed to show any significant differences. None of the 3 groups in this experiment diplayed any correlation between body weight at implant and extent of pulmonary metastasis.

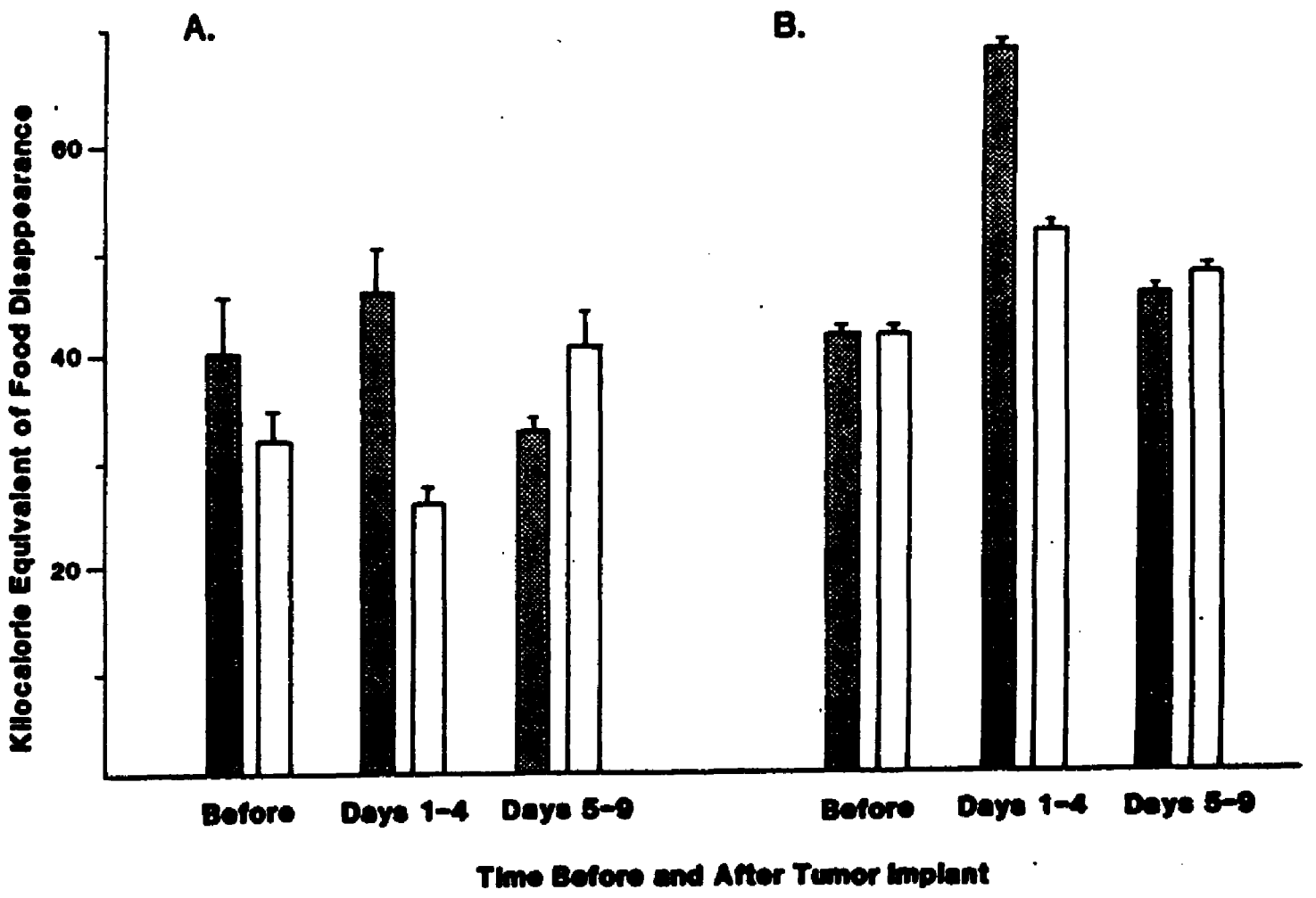
In experiment 3 (Figure 1), both groups switched from RC to the corn oil diets gained about 20 g in the 2 week period following tumor implant (Figure 4, panel C). Throughout the experiment, there was no evidence that the 2 diet groups differed significantly with respect to body weight. Again, there was no significant correlations between body weight at implant and the extent of pulmonary metastasis in either diet group.

The amount of food consumed by 3 rats in each dietary group on the short-term pre-feed protocol (comparable to experiment 1, Figure 1) was measured daily as food

disappearance. Rats were housed individually and records were kept for 3 days before and 9 days after tumor implant. Care was taken to account for food pellets in the bedding. In the HF→LF group, the kilocalorie equivalent eaten during the pre-feed period was approximately 32 kcal/day/rat; this consumption dropped to about 26 kcal/day/rat for days 1-4 after tumor implant as the rats were switched to LF food, then increased to 37 kcal/day/rat during the next 4 day period and stabilized at this level (Figure 5A). This rather large, but transient, drop in consumption has been seen consistently whenever the diet switch has been from HF→LF. The rats appear to favor the HF formulation, and do not accomodate to the LF food for a few days. For those in the LF HF group, the approximate kilocalorie consumption began at 40 kcal/day/rat during the pre-feed period, increased slightly during the first 4 days on the HF food, then returned to 41 kcal/day/rat for days 5-9 and thereafter (Figure 5A). Thus the greatest difference in kilocalorie consumption between the groups occurs during the first 4 days after diet switch and tumor implant, with the HF→LF rats consuming almost 20 kcal/day/rat less than the LF→HF rats.

On the Experiment 2 regimen where rats were fed semi-purified diets from weaning, then switched at the time of tumor implant, food disappearance before and after diet switch was monitored as described above. Rats on the HF diet consumed approximately 32 kcal/day/rat before implant;

Figure 5. Kilocalorie equivalent of food disappearance before and after tumor implant in Experiment 1 and 3. A, food disappearance from cages of retired breeders on short-term feeding protocol (Experiment 1): HF→LF (□); LF→HF (▨). B, food disappearance from cages of retired breeders fed the semi-purified diets at implant (Experiment 1): RC→HF (□); RC→LF (▨). Bars, ± SEM.


A.
B.
Kilocalorie Equivalent of Food Disappearance
60
40
20
Before
Days 1–4
Days 5–9
Before
Days 1–4
Days 5–9
Time Before and After Tumor Implant

after implant and switch to LF diet, only 26 kcal/day/rat were consumed for days 1-4; by days 5-9 the rats resumed eating about 33 kcal/ day/rat (data not shown on graph). This pattern is consistent with the data obtained from the Experiment 1 protocol (Figure 5A) where transfer from a HF to a LF diet resulted in a transient reduction in food intake.

Rats in Experiment 3 were fed RC until tumor implant, then switched to the semi-purified HF or LF diets. Food disappearance was recorded daily for 9 days after tumor implant for 6 rats in each group, housed 3 to a cage. Animals in both groups began to eat substantially more of both of the semi-purified diets immediately after the change of diet; this was particularly noticeable in the RC HF group (Figure 5B). Food disappearance during days 5-9 was comparable in the 2 diet groups (Figure 5B).

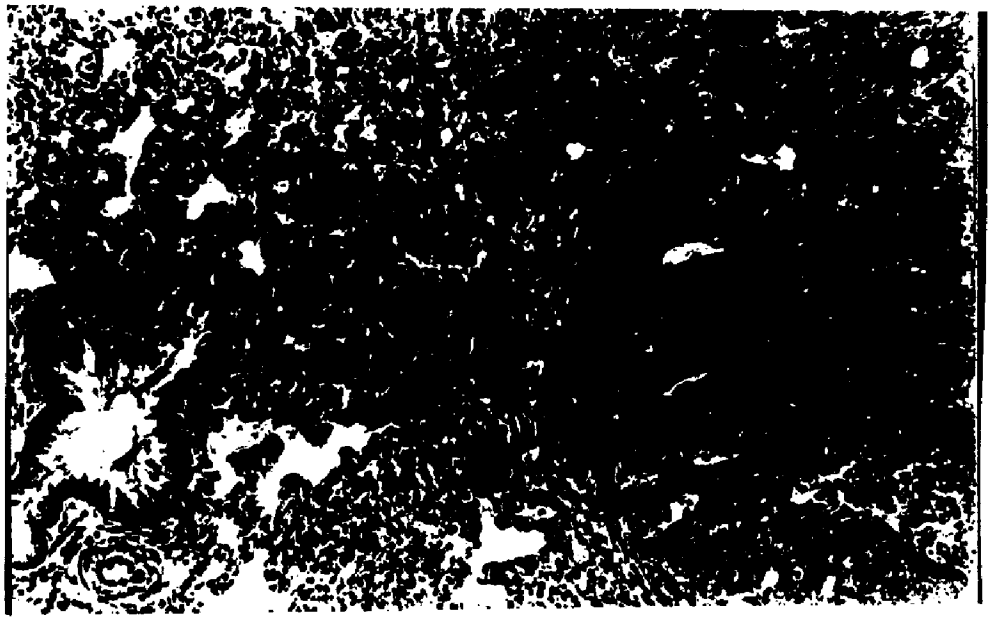
Dietary Effects on Tumor Histology

Representative tumors and metastatic foci from each group in the 3 experiments were examined microscopically. The morphology of the 6 week old tumors in all groups varied little. The periphery of each tumor was characterized by dense clusters of tumor cells separated by stroma. Extensive necrosis occupied the center of these large primary tumors. A portion of a tumor from experiment 1, LF→HF group is shown in Figure 6. Note large,

Figure 6. Primary tumor 6 wk after implant from Experiment 1. The rat had been fed LF for 4 wk prior to tumor implant and then changed to HF for 6 wk between implant and necropsy. Dense clusters of relatively anaplastic tumor cells are separated by stromal elements.

pleomorphic tumor cells with occasional sites where cells form primitive tubules. Essentially the same morphology was found in the foci of tumor cells in the lungs of rats in the various experimental groups. Figure 7 shows a tumor nodule compressing the normal lung tissue adjacent to a bronchiole (experiment 1, HF→HF). These metastatic foci, especially the larger ones, had areas of necrosis and inflammatory infiltrate as did the primary tumors.

Figure 7. Metastatic focus in the lung of a rat from Experiment 1, maintained on HF for 4 wk before and 6 wk after tumor implant. Note similarity of morphology of tumor cells with those in the primary tumor shown in Fig. 6. Left, lung tissue and bronchiole; right, small area of necrosis (arrow) in tumor focus.



DISCUSSION

Data from these experiments provide confirmation of our previous study, demonstrating the stimulatory effect of a high fat corn oil diet on pulmonary metastasis from the 13762 tumor in retired breeder or old virgin female rats (Katz & Boylan 1987, 1987a). Recently, Hubbard and Erickson (1987) reported that lung metastasis from the mouse mammary tumor 4526 was increased when animals were on HF diets high in linoleic acid (principal fatty acid in corn oil) as compared to animals on a HF diet low in linoleic acid. Thus, modulation of metastasis from mammary tumors to distant organ sites has now been demonstrated in two different rodent systems. It is worth noting that Hubbard and Erickson were able to detect a dietary fat effect on metastasis using young mice, 5 weeks old at initiation of the diet using a HF diet differing in linoleic acid content. In contrast, in our rat system with the 13762 MT, no effect of diet on metastasis could be demonstrated in young animals by modulating the amount of fat in the diet (and indirectly the amount of linoleic acid) (Katz & Boylan 1987a; Boylan & Cohen 1986). Whether this age effect holds true for other rat tumor systems should be investigated.

These experiments were designed to determine whether the stimulatory effect of a HF diet on metastasis could be abrogated by transfer to a LF diet at the time of tumor

implant. Reciprocal dietary transfers (LF⟶HF) were also studied. Furthermore, to explore whether a "pre-feed" period using the semi-purified corn oil diets before implant was required, rats were transferred from RC to either HF or LF diet immediately after tumor implant. Data from these 3 experiments clearly demonstrate the importance of acclimation to the semi-purified corn oil diets in the study of the effect of dietary fat on metastasis. Rats maintained on the RC diet before tumor implant failed to show an increased pulmonary tumor burden when fed a HF corn oil diet after implant, indicating that the RC diet (about 5% mixed animal fats, mainly pork lard and beef tallow) had a persistent effect beyond the time of tumor implant. In contrast, if the animals had been pre-fed either one of the semi-purified corn oil diets for 4 weeks before tumor implanntation (as in experiment 1) or had been on the diets since weaning (experiment 2), there was a significant difference between the volume of metastatic tumor found in the lungs of animals fed the HF diet after tumor implant as compared to that of LF-fed animals. These results indicate that a period of feeding the semi-purified corn oil-based diet (either HF or LF) before tumor implantation is necessary in order to show the stimulatory effect of a HF diet on metastasis from the 13762 mammary tumor.

There are a number of possible explanations for the failure of the RC⟶HF protocol to stimulate metastasis. The differences observed between these animals and LF⟶HF

rats were that the latter had gained some weight in the 4 weeks before tumor implantation, and, at tumor implant, they were given a diet with the same type of fat, i.e. corn oil. Since the RB had been pre-fed the semi-purified LF diet for 4 weeks, a large percentage of their body fat was probably composed of triglycerides containing linoleic acid ; thus, the fat pad environment into which the tumor was implanted may have been different from that of rats maintained on a RC diet. This assumption is supported by the work of Herodek and Csakvary (1972) who demonstrated that increased intake of saturated fatty acids resulted in an elevated concentration of oleic in the liver and adipose tissue. Awad (1981) also has shown that the composition of the adipose tissue in rats fed a stock diet (Purina Rat Chow) differs in the amount and types of lipid found when compared to the adipose tissue of rats fed a semi-purified safflower or coconut oil diet. The total lipid content of adipose tissue of rats fed the stock diet (containing 5% fat) was lower than that of rats fed the semi-purified diets (containing 16% fat). The per cent of fat as cholesterol and linoleic acid in triglycerides was also increased in the adipose tissue of rats fed the safflower (polyunsaturated fat) semi-purified diet when compared with the adipose tissue of animals on the stock diet.

The difference in the fat pad environment could also have affected the growth of the primary tumors in the RC HF and RC LF groups, as tumors in both groups tended to be

smaller throughout the experiment than tumors in rats from experiment 1 which had been on the semi-purified diets prior to tumor implant. It is also possible that the other metabolic pathways in the body were unable to respond immediately to the HF corn oil diet after the switch from the RC diet, which, though "low" in fat, contains predominantly saturated animal fats. For example, Spady and Dietschy (1985) have shown that, in the hamster, a saturated fat diet tended to produce higher rates of hepatic cholesterol synthesis than a safflower oil diet. And, Nakano et al. (1971) detected significantly decreased levels of 2 hepatic amino-acid catabolizing enzymes, threonine dehydratase and arginase, in rats switched from a high saturated fat diet to a high carbohydrate diet. Other metabolic adaptations to diets differing in fat content have been recently reviewed by Clarke (1986). Thus, the enzyme systems and serum factors adapted to a saturated fat environment may have had an important, persistent effect on tumor growth and metastasis in the period just after tumor implant and switch to the HF corn oil diet. While we have focused on comparisons on fat quality here, it must be recognized that the RC and semi-purified diets also differ in other respects and that the formulation of RC can vary from batch to batch. Further analysis of this phenomenon must come from comparisons of semi-purified diets containing fats of different origins.

Discussion of the effect of a HF diet on mammary tumorigenesis has recently been focused on whether the promoting effect on tumor incidence or multiplicity is a consequence of increased calories or increased net utilizable energy. Albanes (1987) has reviewed the literature beginning with the classic studies of Tannenbaum in 1940 on mice (1942,1942a) and concludes that "total calorie intake is an important determinant of tumorigenesis in mice". Experiments by Klurfeld et al. (1987) compared the incidence of DMBA-induced MT's in rats placed on a calorie restricted regimen. They concluded that "The tumor promoting effects of dietary fat can be more than offset by a reduction in total caloric intake and .... may be, at least in part, to its greater caloric density." Thompson et al. (1985) have emphasized the element of "net utilizable energy". Induction of MT's by NMU was compared in rats fed HF and LF corn oil diets, restricted in amount to yield the same intake of "net utilizable" kilocalories per day. During the restricted feeding periods (weeks 1-17 after NMU), there was no difference in MT incidence between the HF and LF diet groups. The authors state that their results indicate "the effect of dietary fat.....is not independent of the level of energy intake".

Thus, we were interested in examining our data on diet and metastasis in light of the food consumption and body weights of the rats before and after the transfers between diets. Firstly, if the stimulatory effect of the HF diet

on metastasis was primarily a function of increased calories, the RC HF group in experiment 3 should have shown greatly increased pulmonary metastasis. These rats consumed 45% more calories for the first 5 days on the corn oil diet than rats acclimated to the semi-purified diets before tumor implant, and yet, the extent of pulmonary metastasis was not increased over that of the RC⟶LF group, or other LF⟶LF groups. Secondly, while the groups fed HF after tumor implant in both experiments 1 and 2 showed increased lung tumor burden, the body weights of the rats did not differ significantly from those fed LF diets during the experiments. Thus, if a difference in calories or calorie utilization was involved, its effect must be subtle and not reflected in changes in body weight.

In making these comments, it should be stressed that the end-point we are assessing, i.e. lung metastasis from a transplantable mammary tumor, is very different from that used by Tannenbaum, Klurfeld, Thompson and others, who have been concerned with dietary fat and MT incidence and growth of the primary tumor. In our system, the growth of the primary tumor is not appreciably different in groups on differing diets.

We were struck by the consistent, transient decrease in food consumption in all groups of animals switched from the HF corn oil diet to the LF corn oil diet. This short-term, natural calorie restriction may have played a role in slowing the growth of lung metastases in these

groups; we have experiments in progress to examine this aspect more directly.

Chapter 3

EFFECT OF THE QUALITY OF DIETARY FAT ON TUMOR GROWTH AND METASTASIS FROM A RAT MAMMARY ADENOCARCINOMA

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ABSTRACT

Previously, we reported that a high fat (23% w/w) corn oil diet (HFCO) stimulated metastasis to the lung from the 13762 mammary tumor (MT) as compared to that in rats fed a low fat (5% w/w) corn oil diet (LFCO). This HFCO effect was seen in intact or ovariectomized retired breeders (RB) and in 10+mo old virgins, but not in young virgin rats. Primary tumor growth was comparable in both the high and low fat diet groups. To assess this phenomenon further, we investigated whether the type of fat might affect metastasis from the 13762 MT. Fischer 344 RB were placed into 1 of 5 dietary groups representing mainly polyunsaturated fat (HFCO, LFCO), monounsaturated fat (20% w/w and 5% w/w olive oil, HFOO and LFOO), or saturated fat (20%w/w beef tallow, HFBT). After 4 weeks on the diets, a 2 mm^3 piece of the tumor was implanted sc into each rat. Primary tumor growth and body weight were monitored weekly. Forty days after implant, necropsies were performed, metastatic lung nodules counted, and total metastatic tumor volume calculated. The average volume of pulmonary metastases in the HFCO animals (n=30) was significantly greater than in the other 4 groups. Among the 4 groups which did not differ significantly from each other, the rank order in average volume of pulmonary metastasis was: HFOO (n=25); HFBT (n=26); LFOO (n=25); LFCO (n=18). Growth of the primary tumor did not vary

appreciably among the 5 groups despite the significant difference in pulmonary metastasis volume. The diets vary considerably in fatty acid content; the most salient difference being that the HFCO diet, which stimulated metastasis significantly more than the other diets, contains 4 times more linoleic acid (18:2) than the other diets. The relevance of this difference and other fatty acid differences is discussed. These results suggest that the quality of dietary fat can be an important determinant of pulmonary metastasis from the 13762 MT in RB rats.

INTRODUCTION

Numerous epidemiological and experimental studies implicate dietary fat as a factor in the etiology of breast cancer (Cohen 1986; Gregorio et al. 1985; Miller 1986). Results of experiments using rodent model systems of chemically-induced and transplantable mammary tumors suggest that both the quantity and quality of the dietary fat can influence the incidence and growth of these tumors (Rogers & Wetsel 1981; Gabor et al. 1985; Kort et al. 1987).

Many investigators have found that a diet high in polyunsaturated fat is an effective promoter of mammary tumor growth. This effect seems to be linked to the requirement of essential fatty acids (EFA), especially linoleic acid (18:2), for maximum tumorigenesis. Ip et al. have found that when dimethylbenz(a)anthracene (DMBA) was used to induce mammary tumors in rats fed a 20% fat diet, an EFA concentration in the diet of 4-5% (by weight) was required for maximum tumor incidence and growth (Ip et al. 1985). Kenneth Carroll recently reviewed the effects of different amounts and types of fat on mammary tumorigenesis and concluded that tumor growth is promoted by high levels of dietary fat once the EFA requirement is met, and this additional fat effect seems to be independent of the type of fat (Carroll 1987). However, saturated fatty acids

(main component of beef tallow) and monoenic fatty acids (main component of olive oil) had no specific promoting effects on the growth of the primary tumor (Carroll 1987). Some investigators feel the promotion of tumorigenesis by a high fat diet once the EFA requirement has been met may be due to the increased "net utilizable energy" of a high fat diet (Klurfeld et al. 1987; Thompson et al. 1985).

All of these studies have focused on the effects of dietary fat on chemically-induced primary tumors. Recently, the possible link between dietary fat and metastasis has begun to be investigated. In 1987, we reported that a high fat corn oil diet (mainly 18:2 and other polyunsaturated fatty acids) stimulated pulmonary metastasis in 8-12 month old retired breeder rats bearing the 13762 transplantable mammary adenocarcinoma (Katz & Boylan 1987a). Subsequently, Hubbard and Erickson, using a mixture of coconut and safflower oil, made high fat diets (20%, w/w) containing 1,2,4,8 or 12% 18:2. They showed that a high fat diet with a high percent (by weight) of linoleic acid stimulated pulmonary metastasis from the 4526 transplantable mammary tumor in young mice (1987).

Having shown that the quantity of corn oil (~60% 18:2) can modulate metastasis in our system, we have conducted experiments to address the question of whether different types of fat might affect the process of metastasis to different extents. We used five different diets [high and low fat corn oil (HFCO and LFCO), high and

low fat olive oil (HFOO and LFOO), high fat beef tallow (HFBT)] differing in type and amount of fat. Our results indicate that none of the types of fat in the diet affected the growth of the transplanted primary tumor. However, only the HFCO diet with a very high 18:2 content resulted in a stimulation of pulmonary metastasis from the 13762 mammary tumor.

MATERIALS AND METHODS

Animals and Diets

Female Fischer 344 retired breeder rats (RB), age 10-12 months, were purchased from Charles River Breeding Laboratories, Wilmington, MA. All animals were housed 3 to a cage in a temperature and light-controlled room. The animals were fed one of five semi-purified diets; 1) High fat corn oil (23% w/w), 2) Low fat corn oil (5% w/w), 3) High fat olive oil (20% w/w), 4) Low fat olive oil (5% w/w), 5) High fat beef tallow (20% w/w). The semi-purified diets were obtained from Bio-Serv, Inc., Frenchtown, NJ, in pellet form, stored in the dark at 4°C, and used within 4 weeks. The diets were isocaloric and although the LF diets were not balanced nutritionally with the HF diets, all of the diets contained more than the required amounts of micronutrients. The composition of the HF and LF diets was based on the HFCO and LFCO diets reported earlier (Katz & Boylan 1987a). The diets were fed for a four week period before and six weeks after tumor implantation. The fatty acid profiles of each diet are shown in Table 1. Group sizes for each dietary group are noted in Table 2. Data from two trials of the experiment were pooled.

Tumor Implantation

Pieces ($2mm^3$) of the transplantable 13762 mammary

Table 1. Fatty Acid Profiles of Semi-purified High Fat and Low Fat Diets[a]

	Grams per Kilogram of Diet				
	HF Corn Oil	LF Corn Oil	HF Olive Oil	LF Olive Oil	HF Beef Tallow
Linoleic 18:2n-6	137.4	29.4	28.4	7.1	30.5[b]
Linolenic 18:3n-3	1.9	.4	~	~	~
Oleic 18:1n-9	62.2	13.3	123.0	30.75	76.0
Palmitic 16:0	26.9	5.75	33.8	8.45	49.5
Stearic 18:0	5.1	1.3	5.2	1.3	31.9
Eicosanoic 20:5	.5	.1	~	~	~
Cis 9 hexa-decanoic	~	~	9.6	2.4	5.5
Other	~	~	~	~	7.0

a - Information obtained from supplier, Bio-Serv Inc., Frenchtown NJ.

b - Supplemented with corn oil to provide 3% 18:2 in the diet.

Table 2. Average Pulmonary Metastasis, Body Weights, and Tumor Weights of Rats in the Five Dietary Groups.

	Pulmonary Metastasis (mm^3)		Body Weights (gms ± SEM)			Tumor Weights (gms ± SEM)
	Mean (±SEM)	Median	Initial Weight	At Implant	At Sacrifice	
HF Corn Oil[a] (n = 30)	268.8 ± 88.0[b]	78.7	229 ± 3[c]	259 ± 4[d]	243 ± 5[c]	28 ± 3[c]
LF Corn Oi (n = 18)	15.8 ± 5.0	3.2	226 ± 6	246 ± 5	235 ± 7	27 ± 4
HF Olive Oil (n = 25)	131.6 ± 90.7	14.5	229 ± 3	258 ± 3	246 ± 5	30 ± 3
LF Olive Oil (n = 25)	46.1 ± 13.5	19.3	231 ± 2	246 ± 3	233 ± 4	30 ± 3
HF Beef Tallow (n = 26)	119.3 ± 58.9	17.6	225 ± 4	258 ± 4	245 ± 5	29 ± 3

[a] HF = high fat; LF = low fat
[b] HFCO group had significantly more pulmonary metastasis than all of the other groups ($p \leq 0.05$).
[c] No significant differences among diet groups.
[d] Only HFCO and LFCO groups differed significantly from one another ($p \leq 0.05$).

adenocarcinoma were implanted sc into each rat just posterior to the fourth nipple. The tumor line was obtained through Dr. A. Bogden, Mason Research Institute, Worcester, MA, from the Animal and Human Tumor Bank supported by the Breast Cancer Program, National Cancer Institute, Bethesda, MD. For each experiment, the pieces of tumor for implantation were obtained from 1 rat bearing a tumor transplanted approximately 2 weeks earlier. Details of the transplantation procedure have been reported previously (Katz & Boylan 1987a).

Observation Schedule and Necropsy

In each trial, tumor growth was monitored twice a week with calipers to record two diameters of each tumor. Data on tumor size were expressed as the geometric mean of the tumor diameters ($\sqrt{d \times d}$). Body weights were also taken twice a week.

All rats were sacrificed with CO_2 six weeks after tumor implantation, and necropsies performed. Wet weights of the body and tumor, and site and extent of metastases were recorded.

Data Calculations and Analysis

The extent of the metastasis was determined according to the technique of Welch et al. (1983), which yields an estimate of the total tumor volume of metastatic nodules visible on the lung surface. Metastases with diameters

under 1 mm are assumed to have a radius of 0.5; those with diameters from 1 to 3 mm are assigned a radius of 1.5 mm, and those over 3 mm in diameter are measured individually and their radii figured. Individual tumor volumes are then summed to produce a value of lung tumor volume for each animal.

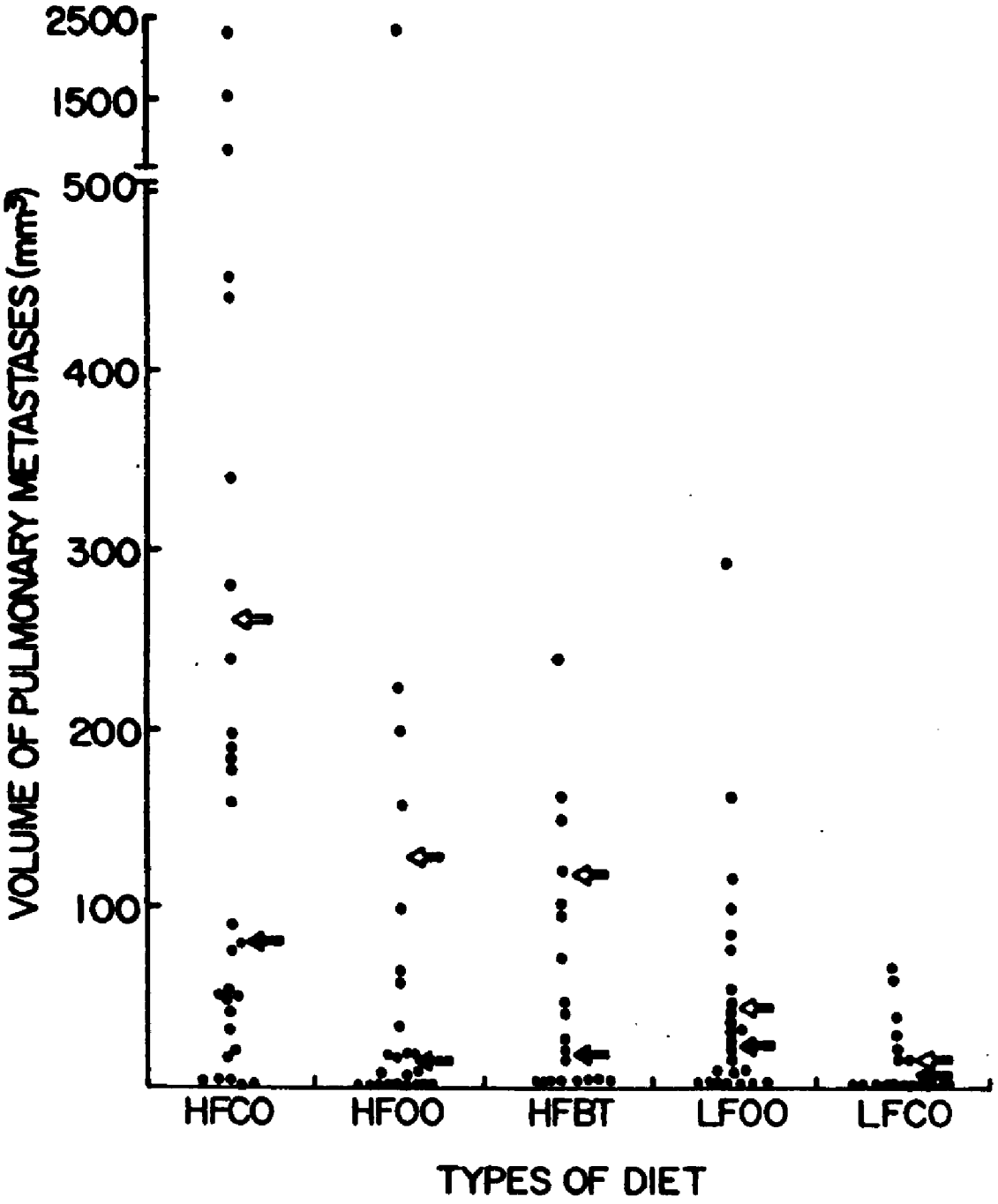
Analysis of the total metastatic tumor volume data was done on log 10 transformed data using the tests of the general linear models on SAS (1986). The tumor growth, body weights, and tumor weights were also analyzed using the tests of the general linear models on SAS (1986).

RESULTS

At necropsy, the lungs of each animal were examined for metastatic foci and the total metastatic tumor volume was calculated. Figure 1 and Table 2 summarize the results. As found in our previous experiments (Katz & Boylan 1987a), the average pulmonary metastatic tumor burden of the HFCO animals was significantly greater than that found in the LFCO rats. The HFCO animals also had significantly more average metastatic tumor in their lungs than in the lungs of the HFOO, HFBT, or LFOO animals. The LFCO, HFOO, LFOO, and HFBT dietary groups did not differ significantly among themselves in the amount of pulmonary metastasis found.

The weights of the animals in each diet group were taken throughout the experiment and are summarized in Table 2. Upon entry into the experiment, rats were switched from a standard laboratory chow diet to one of the 5 semi-purified diets; body weights did not differ significantly among the 5 diet groups at this point. The animals in all of the groups gained weight steadily throughout the 4 week pre-feed period; weights in the four out of five groups did not differ significantly at the time of tumor implant (Table 2). The exception was that the average weight at implant of the LFOO rats (the lowest of the 5 groups) was significantly different from that of the HFCO animals (the highest). Six weeks later at sacrifice,

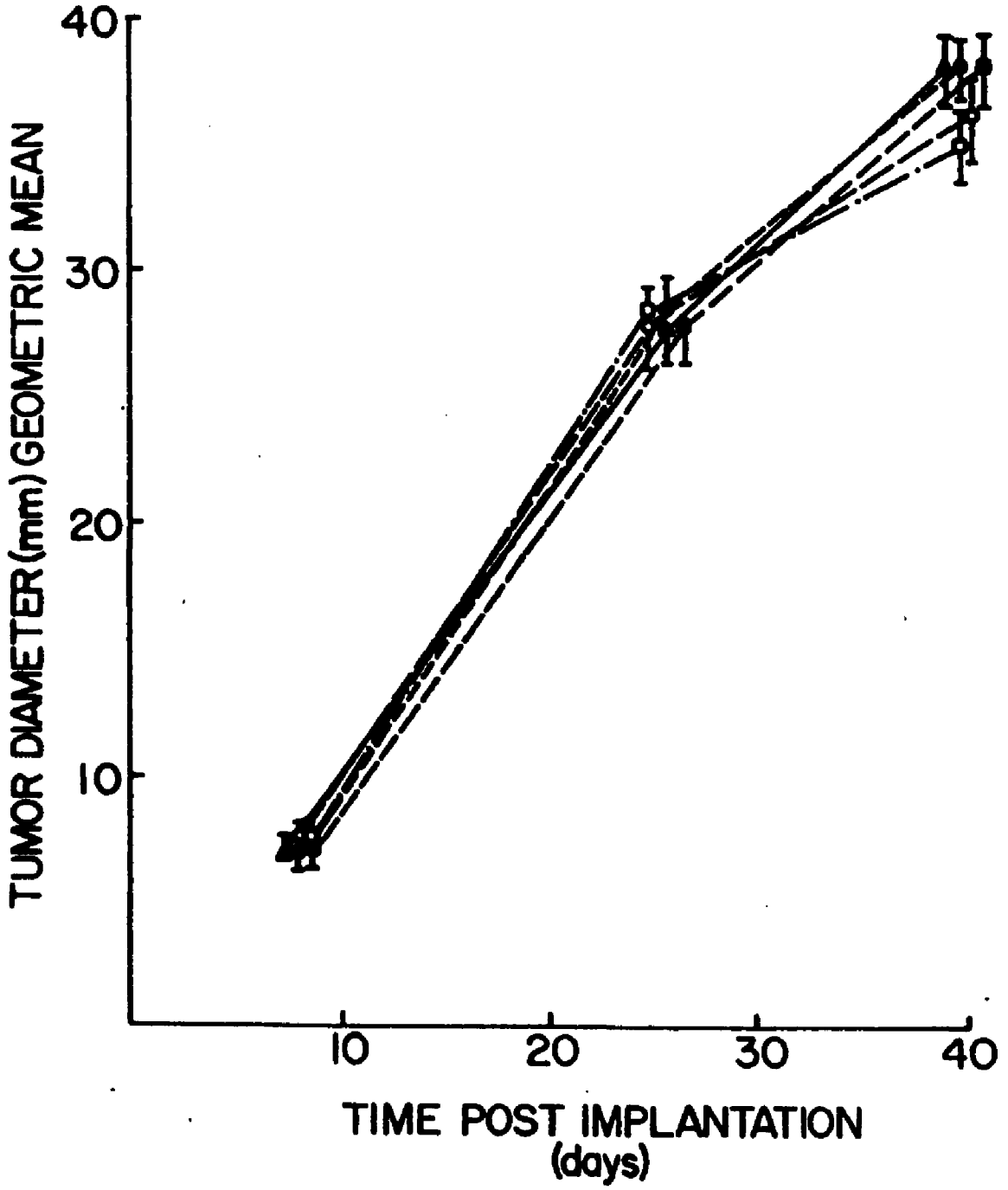
Figure 1. Dots represent the total volume of pulmonary metastases in individual rats in each dietary group after 6 weeks of tumor growth. HFCO = high fat corn oil (23%); HFOO = high fat olive oil (20%); HFBT = high fat beef tallow (20%); LFOO = low fat olive oil (5%); LFCO = low fat corn oil (5%). Open arrows denote mean of each group. Closed arrows denote median of each group.


2500
1500
500
400
300
200
100
VOLUME OF PULMONARY METASTASES (mm³)
HFCO
HFOO
HFBT
LFOO
LFCO
TYPES OF DIET

the average weights again did not differ significantly; however, both groups on the low fat diets did weigh slightly less.

After implantation of a 2 mm^3 piece of tumor, measurable tumors appeared in most rats by 7 days. Primary tumor growth was measured biweekly and the average tumor diameter recorded. The growth of the primary tumors in each diet group was very similar (Figure 2). No significant differences were found among the different dietary groups. At necropsy, the primary tumors were excised and the wet weights determined (Table 2). The average wet weights of the tumors were also very similar and did not vary significantly among the five diet groups. Neither final tumor diameter nor tumor wet weight correlated with the amount of pulmonary metastasis found among rats in the five dietary groups.

Figure 2. Average geometric mean (± SEM) of tumor diameter of RBs in different dietary groups at selected time points. HFCO, n=30 (○); HFOO, n=25 (●); HFBT, n=26 (▲); LFOO, n=25 (■); LFCO, n=18 (□).


40
30
20
10
TUMOR DIAMETER (mm) GEOMETRIC MEAN
10
20
30
40
TIME POST IMPLANTATION
(days)

DISCUSSION

The results of our experiments suggest that both the amount and type of fat in the diet can affect metastasis from the 13762 mammary tumor in retired breeder rats. The HFCO diet was significantly more effective in stimulating metastasis to the lungs than either the LFCO or LFOO diets. Furthermore, while the HFOO and HFBT diets contained virtually the same amount of fat as the HFCO diet, the average amount of pulmonary metastasis found in the lungs of these animals was significantly less than that found in the HFCO rats.

The diets used vary considerably in their fatty acid composition (Table 1). This variation could have contributed to the significant differences in pulmonary metastasis among the different dietary groups. One of the most obvious differences is in the amount of linoleic acid (18:2) found in each diet. The HFCO, which stimulated metastasis to the greatest extent, contains 13% 18:2 (w/w) as compared to about 3% (w/w) in the HFOO, HFBT, and LFCO diets and 0.7% (w/w) in the LFOO diet. The experiments of Hubbard and Erickson (1987) provide direct evidence which illustrates the effect on metastasis of various amounts of 18:2 in high fat diets . They fed high fat diets containing 1, 2, 4, 8 or 12% (w/w) 18:2 to young BALB/c mice with the 4526 mammary tumor inoculated into the mammary fat pad. They found that a diet containing 12%

18:2 stimulated pulmonary metastasis significantly more than the other diets.

The mechanisms by which an increased amount of 18:2 in a high fat diet might stimulate metastasis are varied. The amount of 18:2 in the diet is known to affect the composition and fluidity of tumor cell (Hubbard & Erickson 1987; Liepkalns & Spector 1975) and host immune cell membranes which could affect the functions of these cells (Leung & Ip 1986; Thomas & Erickson 1985). Metastasis could also be aided at several points in the process by the effects of dietary fat on prostaglandin and leukotriene (eicosanoid) production by the host and tumor cells. The modulation of eicosanoid production might affect metastasis by suppressing the immune system's ability to kill tumor cells and the retard the dissemination of metastatic cells (Johnston 1985; Tisdale 1983).

Unlike our results which show an increase in the extent of pulmonary metastasis with an increased amount of 18:2 in the diet, there was no correlation of tumor growth rate (Figure 2) or tumor wet weight (Table 2) at necropsy with the amount of 18:2 consumed. The LFOO animals whose diet included only 0.7% 18:2 (w/w) and the HFCO animals who consumed 13% 18:2 (w/w) had tumor growth rates and tumor wet weights which were very similar and not significantly different from each other. These results imply the minimum 18:2 required for maximum growth of the 13762 mammary tumor in retired breeders is much less than the 4-5% essential

fatty acids which Ip et al. (1985) demonstrated was required for maximum growth of chemically-induced tumors in younger animals. Our results also indicate that this minimum EFA requirement does not have to be in a high fat diet in order for maximum tumor growth to occur, i.e. LFCO and LFOO tumors approximated HFOO and HFBT tumors in growth rate and final size.

Varying amounts of other fatty acids could also have contributed to the differences in the amount of metastasis among the five dietary groups (Table 2). The HFOO diet contains much more oleic acid, a monoenic fatty acid, and the HFBT diet contains appreciably more stearic acid and palmitic acid, saturated fatty acids, than the HFCO diet. Our results indirectly suggest that increasing the amounts of oleic acid or stearic and palmitic acids did not stimulate metastasis from the 13762 MT. Experiments where retired breeders would be fed diets varying in the amounts of these fatty acids and containing a level of 18:2 similar to the HFCO would address the question of whether oleic, stearic or palmitic acids have any specific effects on metastasis from the 13762 MT.

Finally, even though epidemiological evidence shows that there is a positive correlation between age-adjusted breast cancer mortality and percentage of dietary calories as fat, there is no correlation between mortality and percent of calories as polyunsaturated fat (FAO: FAO Monthly Bull. Statist. 1982). Our studies which show that

a high polyunsaturated fat diet (HFCO) stimulates metastasis from the 13762 MT as compared to an isocaloric low polyunsaturated fat diet (LFCO) seem not to agree with these epidemiological studies. But perhaps, as for the growth of chemically-induced tumors, there is a minimum requirement for 18:2 for maximum stimulation of metastasis to occur. This minimum requirement could be more than that required for maximum tumor growth. Carroll (1986) has suggested that every country seems to meet the EFA requirement for maximum tumor growth. He concludes that this supports the idea that it is the total fat consumption which is the important factor in determining mortality from breast cancer. It seems that the threshold amount of 18:2 must be combined with a high fat diet in order for maximum metastasis and thus, increased mortality, to occur. This hypothesis could be tested in our system by feeding the retired breeders a relatively low fat, high carbohydrate diet and a high fat, low carbohydrate diet which contain the same high amount of 18:2 and assess the effect of these diets on the metastasis from the 13762 MT.

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