Breast Cancer Risk in Rats Fed a Diet High in n-6 Polyunsaturated Fatty Acids During Pregnancy

Leena Hilakivi-Clarke, Ighovie Onojafe, Margarita Raygada, Elizabeth Cho, Robert Clarke, Marc E. Lippman*

Background: Women who took the synthetic estrogen diethylstilbestrol during pregnancy exhibit an elevated risk of breast cancer, whereas those who suffered from preeclampsia, which is associated with low circulating pregnancy estrogens, exhibit a reduced risk. Since a high-fat diet may increase circulating estrogen levels and possibly breast cancer risk, dietary factors during pregnancy could influence the risk of developing this disease. Purpose: We tested the hypothesis that consumption of a high-fat diet during pregnancy increases carcinogen-induced mammary tumor incidence in rats. Methods: Pregnant or virgin female Sprague-Dawley rats that had been previously treated with 10 mg 7,12-dimethylbenz[a]anthracene (DMBA) by oral gavage when 55 days old were assigned to one of two isocaloric diets containing either 16% calories from fat (low-fat) or 43% calories from fat (high-fat) for the length of pregnancy or for the equivalent time of approximately 21 days. There were 20 pregnant and 10 nonpregnant DMBA-treated rats per group. Ten additional pregnant animals (not previously treated with DMBA) per group were used for hormone analysis. The fat source used was corn oil, which is high in n-6 polyunsaturated fatty acids, primarily linoleic acid. The animals were checked for tumors at least once per week by palpation. The tumor size, number, and latency to appearance after carcinogen exposure were recorded. The statistical significance of observed differences was tested by use of appropriate two-sided tests. Results: Female rats on different diets had virtually identical food intakes and weight gains during pregnancy. On gestation day 19, serum estradiol levels were approximately twofold higher in rats fed a high-fat diet than in rats fed a low-fat diet (P<.02). The serum insulin levels and insulin/glucose ratios (an index of insulin resistance) in rats fed the high-fat diet were approximately twofold lower than in rats fed the low-fat diet, but the differences did not reach statistical significance (P<.09 and P<.09, respectively). On week 18 following DMBA administration, the number of rats developing mammary tumors was significantly higher in the group exposed to a high-fat diet (40% of animals) than in the group exposed to a low-fat diet (10% of animals) during pregnancy (P<.05). Tumor multiplicity, latency to tumor appearance, and size of tumors upon first detection were similar among the dietary groups. No intergroup differences in the mammary tumor incidence were noted in virgin animals that were exposed to the high- or low-fat diets for an equivalent period of time. Conclusions: Our findings indicate that consumption of a diet high in fat (primarily in the form of n-6 polyunsaturated fatty acids) during pregnancy increases the risk of developing carcinogen-induced mammary tumors, possibly by increasing the pregnancy levels of circulating estrogens. Implications: If further studies find that the results from animal model studies are applicable to humans, some human breast cancers may be preventable by dietary manipulations during pregnancy. [J Natl Cancer Inst 1996; 88:1821-7]
shown that a high-fat diet increases and a low-fat diet reduces the levels of circulating estrogens.

The role of dietary fat intake in breast cancer is controversial. The n-6 polyunsaturated fatty acid (PUFA) linoleic acid has been strongly implicated as a promotional agent in both car-
cinogen-induced and spontaneous rodent mammary tumor models (10,11). In humans, international comparisons and case-
control studies (12) have suggested a relationship between total fat consumption and elevated risk for breast cancer. However,
most cohort studies on breast cancer have found either a border-
line or no relationship (13). None of the previous human or animal studies have investigated the role of dietary fat exposure
during pregnancy. Thus, an important period in life, when the mammary gland may be sensitive to the effects of a high-fat
diet, could have been overlooked.

Until recently, relatively low weight gain and restricted con-
sumption of meat and fat were recommended for pregnant
mothers (14-16). This recommendation was made primarily to
facilitate delivery by producing healthy but relatively low-birth-
weight babies. The emphasis of the prenatal diet was changed in the 1950s, when children born to mothers exhibiting low gesta-
tional weight gain were noted to be at an increased risk of infant
mortality and many developmental delays and abnormalities
(17). The dramatic increase in breast cancer incidence after the
1960s (18) closely followed the change in dietary fat intake in
pregnant women (19-21).

In this study, we tested the hypothesis that exposure to a high-
fat diet during pregnancy may influence carcinogen-induced
mammary tumors in a rat model of breast cancer.

Materials and Methods

Carcinogen Exposure

Seven-week-old female Sprague-Dawley rats were purchased from Charles
River (Wilmington, MA). The rats were administrated 7,12-dimethylbenz[a]anthracene (DMBA) to induce mammary tumors. While DMBA is an experimental carcinogen rather than a causative factor for human breast cancer, it produces mammary tumors that are comparable to those in humans in terms of their long relative latency, histotypes, and endocrine responsiveness (22). At the age of 55
days, a total of 68 female rats were treated with 10 mg of DMBA (Sigma Chemical Co., St. Louis, MO) by oral gavage. The carcinogen was dissolved in peanut oil and given in a volume of 1 mL. The animals were housed in a tempera
ture- and humidity-controlled room at the Georgetown University Re-
search Resource Animal Facility under a 12-hour light-dark cycle. All animal
procedures were approved by the Animal Care and Use Committee of George
town University, and the experiments were performed following the National In-
institutes of Health guidelines for the proper and humane use of animals in
biomedical research.

Pregnancy

Terminal end buds in the mammary gland differentiate to alveolar buds and
lobules during pregnancy, and these differentiated epithelial structures do not
give rise to DMBA-induced cancers (22). Thus, a parous animal treated with
DMBA does not develop mammary tumors. A female rat that is first treated with
DMBA and then undergoes pregnancy, however, will develop mammary tumors.
In the present study, the animals were first treated with DMBA and then made
pregnant.

When the female rats were 75 days old, 48 of them were bred with male rats
(group 1—pregnant), and 20 were housed with females only (group 2—virgin).
Those bred with males were housed as two females together with one male. The
males were kept with the female rats until a few days before the litters were
delivered. Thereafter, the females were housed individually with their offspring.

The offspring were weaned 3 weeks after birth, and the mothers were rehoused
in groups of three or four. The virgin females also were housed in groups of
three or four.

Dietary Manipulations

Upon arrival at our laboratory, the animals were fed Purina Rodent
Laboratory Chow 5001, which contains 12% calories from fat (saturated and un-
saturated). The physiologic caloric value of this diet is 3.3 kcal/g. When the
female rats were housed together with the males (group 1—pregnant) or left in
cages containing females only (group 2—virgin), they were introduced to diets
that had a high (43% calories from fat) or a low (16% calories from fat) fat con-
tent. The fat was derived from corn oil, which contains 59% of n-6 PUFA
linoleic acid. Twenty females in the high-fat group and 20 females in the low-fat

group became pregnant, and there were 10 females in each of the unmated
groups. Animals were fed food and water ad libitum. The diets were within the
range of fat consumed by North Americans; an average North American woman
consumes 37% of calories from fat (23). By comparison, North American
vegetarians consume significantly fewer calories from fat (24), with levels as
low as close to 20% achieved in low-fat dietary intervention studies (25).

The semipurified animal diets were prepared commercially by Bioserv Inc.
(Frenchtown, NJ) in accordance with the guidelines of the American Institute of
Nutrition (AIN) (26) (Table 1). We made the diets isocaloric by adjusting their
fiber content. We adjusted the proportion of dietary components other than fat to
ensure an adequate intake of protein (casein), vitamins, and trace elements, and
the amounts of these components per diet were approximately constant with
regard to energy. On the day the offspring were born, the special diets were
switched back to the standard laboratory diet. The virgin female rats (group 2)
were removed from the special diets at the time that the pregnant females (group
1) began to give birth. Therefore, the animals in both groups were fed the special
(i.e., high- or low-fat) diets for 3 weeks (approximately 21 days).

Hormonal Assays

The effect of diet on serum hormone levels was studied by use of a separate
group of 20 female Sprague-Dawley rats that arrived in our laboratory on day 7
of gestation. Immediately upon arrival, 10 of these rats were placed on the high-
fat diet (i.e., containing 43% of the total calories from corn oil), and 10 were
placed on the low-fat diet (i.e., containing 16% of the total calories from corn
oil). The animals in these two experimental groups were not exposed to DMBA.
Both the various estrogens and total estrogen levels are closely similar in preg-
nant rodents and pregnant humans (27,28). Thus, the total E2 levels have been
found to increase throughout pregnancy in rats and to reach their highest levels
before parturition. To determine the levels of total circulating E2, we collected
blood from five animals per group on gestation days 12 and 19.

Table 1. Dietary formulations

<table>
<thead>
<tr>
<th>Ingredients*</th>
<th>Diet</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low-fat</td>
<td>High-fat</td>
<td></td>
</tr>
<tr>
<td>Fat—total (corn oil), g</td>
<td>70</td>
<td>194</td>
<td></td>
</tr>
<tr>
<td>Protein (casein, L-cystine), g</td>
<td>203</td>
<td>203</td>
<td></td>
</tr>
<tr>
<td>Carbohydrates, g</td>
<td>629.5</td>
<td>340</td>
<td></td>
</tr>
<tr>
<td>Cornstarch</td>
<td>397.5</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>Maltose</td>
<td>132</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Fiber (alaceel), g</td>
<td>50</td>
<td>215.5</td>
<td></td>
</tr>
<tr>
<td>AIN mineral mix, g</td>
<td>35</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>AIN vitamin mix, g</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Choline chloride, g</td>
<td>2.5</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>TBHQ, g</td>
<td>0.014</td>
<td>0.014</td>
<td></td>
</tr>
<tr>
<td>Total, g</td>
<td>1000</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td>kcal density/g</td>
<td>3.72</td>
<td>3.75</td>
<td></td>
</tr>
<tr>
<td>% kcal from fat</td>
<td>15.7</td>
<td>43.2</td>
<td></td>
</tr>
<tr>
<td>% kcal from protein</td>
<td>20.5</td>
<td>19.2</td>
<td></td>
</tr>
<tr>
<td>% kcal from carbohydrates</td>
<td>63.8</td>
<td>37.6</td>
<td></td>
</tr>
</tbody>
</table>

*AIN = American Institute of Nutrition; TBHQ = tertiary butyl hydroquinone; kcal = kilocalories.
In addition to E\textsubscript{2} levels, serum insulin and glucose levels were determined from the same female rats. The animals were not fasted, which induced variability into the insulin and glucose levels. The diets, however, were isocaloric, and the animals in the different dietary groups had similar food intakes (see "Results" section). Our rationale for measuring serum insulin levels was fourfold. First, dietary fat stimulates insulin release from the pancreas. Second, insulin is among the most critical biologic factors associated with normal and malignant growth of mammary tissue (29,30). For example, deprivation of insulin by destroying pancreatic insulin-producing beta cells reduces the growth of DMBA-induced mammary tumors in female rats (31). Third, high levels of insulin are present in colostrum and milk (32). Finally, it is known that high insulin levels during pregnancy are associated with subsequent cardiovascular diseases, hypertension, and diabetes in the mother and her offspring (33-35), suggesting that circulating insulin levels during pregnancy may play a role in altering the risk of developing some diseases.

On the day that the serum samples were collected, the pregnant rats were anesthetized by use of methoxyflurane inhalant, and blood was collected by cardiac puncture between 10 AM and 11 AM. The rats were killed immediately afterward by cervical dislocation. Their blood was placed in tubes, centrifuged at 1000 rpm for 10 minutes at room temperature, and stored at -70 °C. Serum E\textsubscript{2} and insulin concentrations were determined from the samples by use of specific double-antibody kits from ICN Biomedicals, Inc. (Irvine, CA), according to the manufacturer's instructions. Glucose levels were determined by a glucometer (Beckman Instruments, Inc., Fullerton, CA).

### Determining Mammary Tumor Incidence

The rats were checked regularly for mammary tumors by palpation at least once per week. The end points for data analysis were latency to tumor appearance, the size of the tumor upon detection, and the number of tumors. To determine the tumor size, we immobilized the animal by holding it in a firm grip with one hand and with the other hand measuring the length, width, and height of the tumor with the use of a caliper. The rats were killed when the detectable tumor burden was equivalent to approximately 10% of total body weight. The surviving animals and the animals that did not appear to develop mammary tumors were killed 18 weeks after the administration of DMBA.

### Statistical Analyses

Statistical tests were performed by use of the SOLO software (BMDP Statistical Software, Los Angeles, CA). One- or two-way analysis of variance (ANOVA) (36) was used to analyze results for the body weight, food intake, and other parameters associated with pregnancy, hormonal data, and data for tumor size and latency. Where appropriate, between-group comparisons were performed using Fisher's least significant difference test. The logrank test was used to analyze tumor incidence for the two groups. In addition, the chi-squared test was used to analyze the difference in tumor incidence at the last week of tumor measurements (week 18). All statistical tests were two-sided.

### Results

#### Pregnancy

The food intake and body weight gain of the pregnant rats were equivalent in each group. Thus, the pregnant animals kept on a high-fat diet consumed 20.9 g ± 0.3 g of food per day and 77.6 kcal ± 1.0 kcal per day (mean ± standard deviation of the mean [SEM]), and the animals kept on a low-fat diet consumed 20.6 g ± 0.2 g of food daily and 77.2 kcal ± 0.6 kcal per day. The body weights increased from 245.5 g ± 3.6 g (mean ± SEM) at the beginning of pregnancy to 335.8 g ± 9.2 g on day 20 of pregnancy in the high-fat group and from 251.6 g ± 4.1 g at the beginning of pregnancy to 344.2 g ± 8.5 g on day 20 of pregnancy in the low-fat group. Furthermore, we will report, the duration of pregnancy and the number of pups born and their body weights did not differ among the groups (data not shown).

### Effects of Diet on E\textsubscript{2}, Glucose, and Insulin Levels During Pregnancy

The serum levels of E\textsubscript{2}, glucose, and insulin were measured in dietary manipulated female rats on days 12 and 19 of gestation. The E\textsubscript{2} levels were higher on gestation day 19 than on day 12 (two-way ANOVA: \( F = 4.53; df = 1, 14; P<.05 \)). Furthermore, on gestation day 19, the plasma levels of total E\textsubscript{2} were significantly (approximately twofold) higher in the pregnant females fed a high-fat diet when compared with the pregnant females fed a low-fat diet (Student's \( t \) test: \( t = 3.08; df = 8; P<.02 \)) (Fig. 1). On day 12, when the females had been on the special diets for only 5 days, no differences in the E\textsubscript{2} levels were seen (Fig. 1).

Serum glucose levels were significantly higher on gestation day 12 than on gestation day 19 (two-way ANOVA: \( F = 6.67; df = 1 \) and 14; \( P<.02 \)) (Fig. 2). There was a nonsignificant tendency for the insulin levels (Student's \( t \) test: \( t = 1.88; df = 8; P<.09 \)) and the insulin/glucose ratio (an index of insulin resistance) (Student's \( t \) test: \( t = 1.91; df = 8; P<.09 \)) to be reduced on gestation day 19 in the pregnant rats that were fed a high-fat diet when compared with levels obtained in 19-day-pregnant rats kept on a low-fat diet. Both these measures were approximately twofold lower in the rats fed the high-fat diet than in the rats fed the low-fat diet (Fig. 2). We have subsequently repeated these experiments and found that, in BALB/c mice that consumed a 43% high-fat diet throughout pregnancy, the serum insulin levels and insulin/glucose ratio were significantly reduced on the 3rd (last) gestation week (data not shown).

### Effects of Diet on Mammary Tumorigenesis

The mammary tumor incidence was significantly higher among the rats fed the high-fat diet during pregnancy than among the rats fed the low-fat diet during pregnancy (logrank test = 4.64; \( df = 1; P<.031 \)) (Fig. 2). Thus, at week 18 after DMBa administration, 40% of the animals fed the high-fat diet had developed mammary tumors, whereas 10% of the animals

![Fig. 1. Serum levels (mean value ± standard error of the mean) of total estradiol in pregnant female Sprague-Dawley rats that were fed isocaloric high-fat (43% calories from fat) or low-fat (16% calories from fat) diets from gestation day 7 onward. Each group contained four or five animals. Asterisk indicates statistical significance at the \( P<.05 \) level.](image-url)
fed the low-fat diet had tumors (chi-squared = 4.80; df = 1; P<.05). Other parameters of tumorigenesis were unaffected by dietary exposure during pregnancy. The latency to tumor appearance, the size of the tumors upon first detection, and tumor multiplicity (number of tumors per animal) were similar in rats exposed to a high- or a low-fat diet during pregnancy (Table 2).

The mammary tumor incidence was not altered among virgin female rats exposed to special diets for a 3-week period (Fig. 3). In the present study, the overall level of mammary tumor incidence in rats treated with DMBA was lower than in our other studies (37). In our experience, DMBA given to 55-day-old rats typically produces a 50% tumor incidence, and those animals that do develop mammary tumors have one to three tumors per animal. In the present study, the rats were handled; they were weighed frequently, and their food intake was measured. Handling rats after DMBA administration has been reported to reduce the incidence of DMBA-induced mammary tumors [see (38)].

Discussion

Our data show that the consumption of a diet high in fat, primarily in the form of n-6 PUFA, by female rats during pregnancy increases their susceptibility to mammary tumorigenesis. Such rats exhibited a significantly higher incidence of DMBA-induced mammary tumors if they were fed a diet containing 43% calories from fat (in the form of corn oil) than if they were fed a diet containing 16% calories from fat (corn oil) during pregnancy. We also generated data indicating that short-term exposure (3-week exposure occurring 21 days after DMBA administration) of virgin animals to a high-fat diet does not alter the incidence of carcinogen-induced mammary tumorigenesis. The lack of an effect by this short-term exposure is in line with the findings of several other investigators [reviewed in (10)]. Although a diet high in linoleic acid generally increases tumorigenesis in carcinogen-induced or spontaneous mammary tumor models, a long rather than a short exposure is required (39). In addition, our data indicate that a diet high in linoleic acid increases tumor incidence if this diet is consumed during pregnancy.

Our finding that pregnancy is a period during which a rat is vulnerable to the tumor-promoting effects of dietary fat may reflect the functional changes occurring in the mammary gland during pregnancy. Two major events occur in the mammary gland during pregnancy. First, the number of epithelial structures increases substantially as a result of rapid cell proliferation (40). Second, terminal end buds and terminal ducts differentiate into alveolar structures in rodents (40). In humans, type 1 lobules differentiate into type 3 lobules (41). The pregnancy-associated changes in the mammary gland structure and function result from the increase in circulating hormone levels (40). Estrogen is a promoter of breast cancer (42), although the exact molecular pathways for its action on mammary epithelial cells have remained unclear. In our present and in another study, we found an increase in the serum levels of total E2 in pregnant rats exposed to a high-fat diet. Since breast cancer risk is elevated in women who were exposed to DES during pregnancy (2) and reduced in women who had low estrogen levels during pregnancy (3), our results obtained in rats may be associated with the fat-induced increase in circulating E2 levels. Estrogens might increase proliferation of the mammary glands during pregnancy, since the role of this hormone is closely linked to mammary

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**Fig. 2.** Serum levels (mean value ± standard error of the mean) of glucose (A), insulin (B), and insulin/glucose ratio (C) in pregnant female Sprague-Dawley rats that were fed isocaloric high-fat (43% calories from fat) or low-fat (16% calories from fat) diet from gestation day 7 onward. Each group contained four or five animals.

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**Table 2.** Mean latency to the appearance of a tumor, area of tumors at first detection, and tumor multiplicity in 7,12-dimethylbenz[a]anthracene-treated rats exposed to a diet containing 16% (low-fat) or 43% (high-fat) calories from fat throughout pregnancy (n = 20 per group) or for 21 days (virgin controls, n = 10 per group).

<table>
<thead>
<tr>
<th>Diet</th>
<th>No. of tumors</th>
<th>Tumor latency, wk*</th>
<th>Tumor area, mm²*</th>
<th>Tumor multiplicity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mated rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low fat</td>
<td>2</td>
<td>9.5 ± 1.5</td>
<td>95.0 ± 5.0</td>
<td>1.0 ± 0</td>
</tr>
<tr>
<td>High fat</td>
<td>8</td>
<td>10.4 ± 0.9</td>
<td>79.5 ± 11.0</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>Unmated rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low fat</td>
<td>3</td>
<td>12.3 ± 2.3</td>
<td>42.7 ± 13.3</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>High fat</td>
<td>3</td>
<td>12.0 ± 0.1</td>
<td>42.0 ± 24.0</td>
<td>1.5 ± 0.5</td>
</tr>
</tbody>
</table>

*Values = means ± standard error for proliferating tumors.
epithelial cell growth (43). Thus, the mammary epithelial cells of pregnant rats that were pretreated with a carcinogen and exposed to elevated E2 levels as a result of a high-fat diet may have been subjected to a higher degree of neoplastic transformation than the mammary epithelial cells of pregnant animals consuming a low-fat diet that results in normal pregnancy E2 levels.

Although E2 is a strong candidate to mediate the difference in mammary tumor incidence between female rats exposed to a high-fat diet during pregnancy and those exposed to a low-fat diet during pregnancy, it is possible that dietary fat was directly involved. For example, a high-fat diet may have induced changes in the structural composition of membranes (44) or metabolic pathways that lead to altered phospholipase C or protein kinase C activity (45-47). Since several of these events are also thought to be regulated by estrogens (48-50), the effects of high dietary fat intake during pregnancy on subsequent mammary tumor incidence could be due to both E2 and fat.

Late pregnancy is characterized by hyperinsulinemia accompanied by insulin resistance (51,52). Since dietary fat stimulates insulin secretion from the pancreas (28), and insulin is linked to both the normal and malignant growth of mammary cells (29,30), we expected the serum levels of insulin to be elevated during pregnancy (logrank = 4.64; p<0.031). Our data show that high dietary intake of essential fatty acids by rats during pregnancy reduces their serum insulin levels. These rats, exposed to a high-fat diet during pregnancy, exhibit an increased incidence of mammary tumors. Thus, low levels of linoleic acid and high levels of insulin during pregnancy appear to protect against breast cancer (human data), while high levels of linoleic acid and reduced circulating levels of insulin during pregnancy increase mammary tumor incidence in rats.

Because of the high epithelial cell proliferation, pregnancy has been thought to be a risk factor for recurrence of breast cancer among breast cancer survivors. A significant proportion of treated breast tumors relapse within 5 years of original diagnosis. However, in the light of many studies (58-60), it is now evident that pregnancy does not pose an additional risk. The data obtained in the present study with an animal model are in support of these findings. The incidence of carcinogen-induced mammary tumors was 30% in the virgin animals and 10% (low fat) and 40% (high fat) in female rats who became pregnant after the carcinogen treatment. Thus, pregnancy that occurred 3 weeks after the carcinogen exposure did not increase breast cancer risk in the female rats.

In conclusion, we found that consumption of a high-fat diet during pregnancy increases the risk in female rats of developing DMBA-induced mammary tumors. Since a high dietary intake of fat was associated with elevated serum E2 levels and previous data in humans suggest that high estrogen activity during pregnancy increases (2) and low estrogen levels reduce (3) breast cancer risk, E2 and E1 may be an important mediator of the increased risk. Therefore, while our data may have important implications for the attempts to prevent some breast cancers by modulating
References


(33) Hilkavicius-Lange L, Wright A, Lippman ME. DMBa-induced mammary tumor growth in rats exhibiting increased or decreased ability to cope with stress due to early postnatal handling or antidepressant treatment. Physiol Behav 1993;54:229-36.


Notes


Supported in part by grant CN-80420 from the American Cancer Society; by a grant from the Cancer Research Foundation of America; and by Public Health Service grants RO1CA58022, P50CA58185, and P30CA51008 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

Manuscript received May 31, 1996; revised August 30, 1996; accepted September 27, 1996.