Effect of Moderate Vitamin A Supplementation and Lack of Dietary Vitamin A on the Development of Mammary Tumors in Female Rats Treated with Low Carcinogenic Dose Levels of 7,12-Dimethylbenz(a)anthracene

Maija H. Zile, Malford E. Cullum, Irene A. Roltsch, Jane V. DeHoog, and Clifford W. Welsch

Departments of Food Science and Human Nutrition and Anatomy, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

We examined the effect of moderately increased and of marginal continued dietary supplementation of vitamin A (retinyl acetate) and the effect of lack of dietary vitamin A on the initiation and promotion stages of mammary tumorigenesis in female Sprague-Dawley rats treated with a single low (0.5 mg/100 g body weight) or very low (0.1 mg/100 g body weight) dose of i.v.-administered 7,12-dimethylbenz(a)anthracene. The number of mammary tumors was significantly (*P < 0.05) reduced if prior to and during initiation with 7,12-dimethylbenz(a)anthracene the rats were fed a moderately increased (30 µg/day) or marginal (3 µg/day) amount of vitamin A, compared to rats fed an adequate (10 µg/day) amount of vitamin A. The number of mammary tumors was also significantly (*P < 0.05) reduced when a moderately increased or marginal amount of vitamin A was provided during the tumor promotion phase. In addition, the number of mammary tumors was significantly (*P < 0.05) reduced by the lack of dietary vitamin A during both the initiation and promotion stages of this tumorigenic process, when compared to vitamin A adequate, ad libitum-fed rats, but not when compared to vitamin A adequate, food-restricted controls. The reduction in numbers of mammary tumors observed in these studies was reflects primarily in significant (*P < 0.05) decreases in mammary fibroadenomas; the number of mammary carcinomas was often reduced, but due to a low frequency of the carcinomatous lesions, this reduction did not reach the 5% level of statistical probability. Plasma and liver vitamin A levels were determined during both the initiation and promotion stages. As the dietary supplementation of vitamin A increased from 0 to 30 µg/day, there was an increase in mean liver and plasma vitamin A levels. No consistent correlation between plasma and liver vitamin A levels and the occurrence of mammary tumors was observed, except with the marginally increased (30 µg/day) intake of vitamin A, that resulted in a small, but statistically significant (*P < 0.05) increase of serum retinol at initiation; this may account for the observed reduction in mammary tumors.

These results provide evidence that moderate alterations in vitamin A consumption can modulate low-dose chemically induced mammary gland tumorigenesis. Most importantly, suppression of mammary gland tumorigenesis can be achieved by moderately increased, frequent, and regular consumption of vitamin A; prolonged consumption of vitamin A-deficient diets or diets marginal in vitamin A does not enhance the risk of mammary tumor development.

INTRODUCTION

The relationship between vitamin A nutrition and cancer has been known for many years (1, 2). One aspect in this relationship is the increased susceptibility of vitamin A-deficient animals to certain epithelial cancers (3-11). More recently a number of epidemiological studies revealed an inverse relationship between the incidence of cancers of lung, bladder, mouth, larynx, cervix, and breast and vitamin A consumption in humans (reviewed in Refs. 12 and 13). Such studies support the experimental work with animals and suggest that diets deficient or inadequate in vitamin A increase the risk of development of certain epithelial tumors. These observations are particularly relevant in view of recent United States and Canadian population surveys demonstrating that as high as one-third of our population has lower than adequate tissue levels of vitamin A (14-18).

It has been amply documented that pharmacological levels of retinoids (natural and synthetic vitamin A compounds) markedly suppress the development of mammary tumors in experimental animals, in particular, chemically induced rat mammary carcinomas (11, 19-24). However, no studies to date have been conducted to determine whether or not experimental animals, fed diets deficient or marginally low in vitamin A, have altered sensitivities to the development of mammary tumors. Similarly, there have been no studies to examine the potentially prophylactic effect of moderately high, continued dietary supplementation of vitamin A on this neoplastic process. To examine these aspects of vitamin A nutrition in tumor prevention, we have chosen to utilize a modified version of the well-known and extensively studied DMBA-induced rat mammary carcinoma model (19-24). We have modified the model only by the utilization of a very low dose of the carcinogen. Such dose levels induce a relatively low yield of mammary carcinomas and a considerably high yield of benign mammary neoplasms over a relatively longer period of time. The tumor development is thus more closely akin to that observed in human populations. The DMBA-induced rat mammary carcinoma model is a logical choice for our studies since the tumors induced by the carcinogen are responsive to vitamin A (19-24) and since the potentially modulating effect of vitamin A can be examined at both the initiation and promotion stages of this carcinogenic process.

MATERIALS AND METHODS

Animals and Diets

Female Sprague-Dawley rats were purchased from Holtzman Co., Madison, WI, at 20 days of age. Animals were individually housed in raised, wire mesh cages in an environmentally controlled room (light, 12 h; temperature, 24°C; relative humidity, 40%). Weights were recorded weekly. Water and a vitamin A-deficient semisynthetic diet were provided ad libitum, except where diet restriction was required. The diet was that of the AIN-76 diet (25) with vitamin A omitted and provided in the following amounts: casein, 20 (ALACID 710; New Zealand Milk Products, Petaluma, CA); dextrose, 50 (Clintose Dex A; Archer Daniels Midland Co., Decatur, IL); corn oil, 5 (Mazola; Best Foods, Union, NJ); mineral mix, 3.5 (AIN-76; United States Biochemical Co., Cleveland, OH); sucrose, 40 (Clintose Sucrose); and mineral mix, 3.5 (AIN-76; United States Biochemical Co., Cleveland, OH). The diet was dextrose substituted for sucrose. Ingredients for the diet (weight per choice for our studies since the tumors induced by the carcinogen are responsive to vitamin A (19-24) and since the potentially modulating effect of vitamin A can be examined at both the initiation and promotion stages of this carcinogenic process.

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2 To whom requests for reprints should be addressed.

The abbreviations used are: DMBA, 7,12-dimethylbenz(a)anthracene; HPLC, high-pressure liquid chromatography; BHT, butylated hydroxyltoluene.

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acetate per day (adequate vitamin A) but body weights maintained within -20°C. Ingredients for the premix (grams) were: thiamin mononitrate, 6; riboflavin, 6; nicotinamide, 30; pyridoxine hydrochloride, 7; d-calcium pantothenate, 16; folate, 2 (Sigma); biotin, 0.2; vitamin B12, 0.01 (Sigma); vitamin E (dl-a-tocopheryl acetate), 100; vitamin D2, 1.18; vitamin K (phytonadione), 0.13; and dextrose, 9831. All diet components, except those from Sigma Chemical Co., were generously donated by their respective sources. Vitamins were a gift from Hoffmann-LaRoche, Nutley, NJ. The casein (ALACID 710) contained 7.6 µg retinyl palmitate per 100 g. Ten g of the diet thus contained 0.2 µg of vitamin A.

Retinyl Acetate Supplements

Crystalline all-trans-retinyl acetate (Hoffmann-LaRoche) was initially dissolved in a small amount of acetone, corn oil was then added, and a nitrogen stream was passed through to remove the acetone. Retinyl acetate concentrations of the supplements were determined at 325 nm in a Beckman Model 35 spectrophotometer. The supplements were prepared to contain 3, 10, and 30 µg retinyl acetate per 0.05 ml oil. Supplements were prepared biweekly and stored at -20°C in opaque vials. The above operations were carried out in a room equipped with gold fluorescent lights (General Electric FG4060). Two-day supplements (0.1 ml) were given to the rats on top of the food 3 times a wk (Monday, Wednesday, and Friday), and a 1-day supplement was given on Sundays (0.05 ml) using an automatic pipet (Microman; Rainin Instrument Co., Inc., Woburn, MA). This method of dietary supplementation was found to be as effective as gastric intubation, since rats consumed the supplements completely and immediately, thus also eliminating the possibility of vitamin A destruction by oxygen and light.

Experimental Design

Over a 3-yr period, three experiments were conducted (Experiments 1, 2, and 3). Each experiment consisted of two studies that were designed to establish (a) whether vitamin A nutrition is important in the prevention of tumor initiation in the rat mammary gland (initiation study), and (b) whether vitamin A nutrition is important in the prevention of mammary tumor development during the promotional phase of mammary gland tumorigenesis (promotion study).

Upon arrival, weanling female rats were randomly assigned to 3 groups of approximately 50 to each study. In the initiation study, the rats were fed the semisynthetic diet (as described above) and supplemented with retinyl acetate as follows: Group A, 3 µg retinyl acetate per day (marginally low vitamin A); Group B, 10 µg retinyl acetate per day (adequate vitamin A); and Group C, 30 µg retinyl acetate per day (moderately increased vitamin A). In Experiment 2, 2 additional groups of rats were included, as follows: Group D, no retinyl acetate (oil vehicle only); and Group E, 10 µg retinyl acetate per day (adequate vitamin A but body weights maintained within 10 g of the mean body weights in Group D, by food restriction, as necessary). This dietary protocol was continued for all groups until 7 days after carcinogen treatment; thereafter all rats received 10 µg retinyl acetate per day.

In the promotion study, the weanling rats in all groups were fed the semisynthetic diet supplemented with 10 µg retinyl acetate per day until 7 days after carcinogen treatment; thereafter the animals began to receive varied amounts of retinyl acetate supplements, as follows: Group A, 3 µg retinyl acetate per day (marginally low vitamin A); Group B, 10 µg retinyl acetate per day (adequate vitamin A); and Group C, 30 µg retinyl acetate per day (moderately increased vitamin A). In Experiment 2, two additional groups of rats were included, as follows: Group D, no retinyl acetate (oil vehicle only); and Group E, 10 µg retinyl acetate per day (adequate vitamin A but body weights maintained within 10 g of the mean body weight in Group D, by food restriction, as necessary). This dietary protocol was continued for all groups until the termination of the study.

Designation of Vitamin A Supplements

The following criteria were used to select the different vitamin A treatments.

Marginally Low Vitamin A, 3 µg/Day. Although an exact maintenance level of dietary vitamin A for the rat has not been established, available data from several studies suggest that a daily dietary supplementation of 2.5 to 5.0 µg of vitamin A results in no liver storage of vitamin A, although the rats grow and develop normally as judged by all external criteria (26-30). This dietary regimen therefore represents a marginally low vitamin A intake for the rat, without a margin of safety for extra metabolic needs.

Adequate Vitamin A, 10 µg/Day. This level of vitamin A supplementation represents a normal vitamin A intake that will result in optimum growth and development of young rats and also will result in some liver storage of the vitamin. The choice of the amount of vitamin A designated as "adequate" and of fulfilling the above criteria is based on several literature sources (27-33). Additionally, the National Academy of Sciences-National Research Council recommendation cites the requirement for rats as 2000 IU (or about 700 µg) of vitamin A per kg of diet; for a female rat, consuming 10 to 15 g of diet per day, the daily recommended requirement thus is 7 to 10.5 µg of vitamin A (25).

Moderately Increased Vitamin A, 30 µg/Day. This level of vitamin A supplementation is considered ample vitamin A nutrition in the rat; it results in optimum growth and considerable liver storage of the vitamin, and it is recommended for full longevity of the rat (29-31, 34). The AIN-76 diet (25), that is widely used in experimental nutrition, contains 4000 IU of vitamin A per kg of diet and provides 13 to 20 µg of vitamin A per day to a rat eating 10 to 15 g of diet. Based on this information and the National Academy of Sciences-National Research Council's vitamin A requirement for the rat, we selected 30 µg of vitamin A per day as a moderately increased dietary amount of vitamin A.

Carcinogen Treatment

Rat mammary tumors were induced with DMBA by a single injection in the tail vein, in an oil emulsion (Upjohn Co., Kalamazoo, MI). In Experiment 1, the rats were treated with a low dose of carcinogen (0.5 mg DMBA per 100 g body weight) at the age of 63 days. In Experiments 2 and 3 a very low dose of DMBA was injected (0.1 mg/100 g body weight) at the ages of 65 and 55 days, respectively. The events associated with mammary tumor initiation were considered to be completed by 7 days after injection of the carcinogen (35).

Detection and Histopathological Evaluation of Mammary Tumors

Tumor palpations began 2 mo after DMBA treatment in Experiment 1 and 1 mo after DMBA treatment in Experiments 2 and 3, and they were performed biweekly. When tumors were about 2.0 cm in diameter, they were surgically excised from the lightly anesthetized rat, fixed in Bouin's solution, stained with hematoxylin and eosin, and histologically examined. These rats were returned to the experiment. At the termination of experiments, all rats were sacrificed, and palpable as well as nonpalpable mammary tumors were excised and examined. The tumors were classified either as carcinomatous or benign (fibroadenomas) mammary neoplasms, utilizing standard criteria as outlined by Young and Hallows (36).

Biological Materials for Determination of Vitamin A

Determination of vitamin A was conducted on biological materials from Experiment 2. Plasma and liver vitamin A values were determined at the start of the experiment in 6 to 7 weanling rats representative of all rats from the various groups. Samples for vitamin A assessment at the time of tumor induction were obtained as follows. One day before carcinogen treatment, 6 to 7 rats from each group of the initiation study were lightly anesthetized with ether, and a liver biopsy specimen about 1 cm in diameter was surgically excised from each rat; 1 ml of blood was obtained by heart puncture. The animals were returned to the experiment. The heparinized blood was centrifuged to obtain plasma. Liver biopsy samples and plasma were immediately frozen on dry ice and stored at -80°C. At the termination of experiments, liver and plasma were obtained from all animals (initiation and promotion studies) and stored at -80°C, in the dark. For the data reported here (Experiment 2), the sera and liver were analyzed in 6 rats from each
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Determination of Vitamin A

A semimicromodification of the Ames method (37), used for retinoid extraction from livers, was as follows. Five hundred mg of anhydrous sodium sulfate were ground with 100 mg of wet liver and extracted for 24 h with 1 ml of stabilized chloroform (0.1% ethanol, 10 µg of BHT per ml), containing 2 to 5 µg of 13-cis-ethylretinamide as internal standard. Four ml of redistilled methanol were mixed with the extract, and 50 µl of the resulting supernatant were injected on the HPLC column. Plasma (0.1 ml) was treated with 2.5 volumes of ethanol containing 20 to 50 ng of 13-cis-ethylretinamide and then extracted 3 times with 10 volumes of hexane. All extraction solvents contained 10 µg of BHT per ml. The solvent was removed by a stream of nitrogen, and the sample was redissolved in 50 µl of methanolchloroform, 85:15, for injection onto a HPLC column.

Quantitation of retinoids was by HPLC as described previously (38). The HPLC equipment consisted of a single pump (M45SC; Millipore Waters, Milford, MA), a Hg lamp UV detector with a 340-nm interference filter (Model 440; Millipore Waters), a reporting integrator (H-F 3390A; Hewlett-Packard, Avondale, PA), and two miniature inert valves (HV PD 4-5; Hamilton Company, Reno, NV) for distribution of solvents. Chromatography was carried out on a C-18 reversed phase column, 25 x 0.46 cm. ODS-2 10/25 (Whatman, Clifton, NJ), protected by a 7 x 0.46-cm guard column containing Co-Pell ODS (Whatman). The column was developed by step gradient elution using methanol:water, 90:10 (v/v), 7 min; followed by methanol: water, 93:7 (v/v), 5.5 min; and methanol:chloroform, 85:15 (v/v), 15 min. Elution was at 2 ml/min. Absorbance was monitored at 340 ± 20 nm, using 0.02 absorbance units full scale. Retinoids from biological samples were quantitated from the integrated peak areas at 340 nm and from the established external standard curves of retinol, retinyl acetate, and retinyl palmitate, as described previously (38); for internal standardization, 13-cis-ethylretinamide was added to samples prior to extraction, and its recovery was monitored by HPLC-UV absorbance. All procedures were conducted under gold fluorescent lights.

Statistical Analysis

Plasma and liver vitamin A values, mean body weights, and mammary tumors per rat were analyzed by one-way analysis of variance. Mean separations were performed using Duncan's multiple range test. x² analysis was used for analysis of mammary tumor incidence and for analysis of total number of mammary carcinomas and fibroadenomas. For mean values to be significantly different, P must be equal to or less than 0.05.

RESULTS

Effect of Retinyl Acetate Feeding on the Initiation Stage of Low Dose Level DMBA-induced Rat Mammary Gland Tumorigenesis. The total number of mammary tumors in female rats fed 3, 10, and 30 µg of vitamin A (retinyl acetate) per day prior to and during a low dose level (0.1 to 0.5 mg/100 g body weight) administration of a mammary gland carcinogen (DMBA) (initiation study) is shown in Table 1. Upon combining the results obtained in Experiments 1 to 3, it was observed that feeding moderately increased (30 µg/day) or marginal amounts (3 µg/day) of vitamin A reduced the number of mammary tumors by 13 and 26%, respectively, when compared to rats fed an adequate amount (10 µg/day) of vitamin A; this reduction in mammary tumor yield was statistically significant. When the mammary tumors were separated according to histopathological type (mammary carcinomas and benign mammary fibroadenomas) (Table 2), it was found that the total mammary carcinoma number (combining Experiments 1 to 3) was reduced by 16% in rats fed moderately increased vitamin A levels, and by 10% in rats fed marginal amounts of vitamin A, when compared to adequately vitamin A supplemented rats; these reductions, however, did not quite reach the 5% level of statistical probability. In rats fed moderately increased amounts of vitamin A and in rats fed marginal amounts of vitamin A, the total number of mammary fibroadenomas (combining Experiments 2 and 3) was reduced by 18 and 37%, respectively, a reduction that was statistically significant. Since the number of rats in each group at the onset of the study was not identical (Experiment 1), a correction was made for group size differences; this correction was incorporated in the computation of the percentage of changes in total mammary tumor number and in the statistical computation of these data.

The mean percentage of rats bearing mammary carcinomas in the groups of rats fed 3, 10, and 30 µg of vitamin A per day was 37, 42, and 32%, respectively (combining Experiments 1 to 3); the mean percentage of rats bearing benign mammary fibroadenomas was 48, 57, and 58%, respectively (combining Experiments 2 and 3) (Table 2). No significant effect of varied dietary vitamin A ingestion was observed on this parameter.

Effect of Retinyl Acetate Feeding on the Promotion Stage of Low Dose Level DMBA-induced Rat Mammary Gland Tumorigenesis. The total number of mammary tumors in female rats fed 3, 10, and 30 µg of vitamin A (retinyl acetate) per day commencing 7 days after the administration of a low dose level of DMBA (promotion study) is shown in Table 3. Upon combining Experiments 1 to 3, it was observed that moderately increased (30 µg/day) or marginal amounts (3 µg/day) of vitamin A reduced the number of mammary tumors by 28 and 43%, respectively, when compared to rats fed an adequate amount (10 µg/day) of vitamin A; this reduction was statistically significant. Upon separating the tumors according to histological type (Table 4), it was observed that the total mammary carcinoma number (combining Experiments 1 to 3) was virtually unchanged (1% increase) in rats fed moderately increased amounts of vitamin A and reduced (15%) in rats receiv-

Table 1 Effect of retinyl acetate administration during initiation on total number of mammary tumors in female rats treated with low dose levels of DMBA

<table>
<thead>
<tr>
<th>Group</th>
<th>Retinyl acetate (µg/day)</th>
<th>Total no. of mammary tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DMBA (mg/100 g body wt)³</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>A, marginal vitamin A</td>
<td>3</td>
<td>104y (47)y</td>
</tr>
<tr>
<td>B, adequate vitamin A</td>
<td>10</td>
<td>107y (53)</td>
</tr>
<tr>
<td>C, moderately increased vitamin A</td>
<td>30</td>
<td>85y (43)</td>
</tr>
</tbody>
</table>

² The indicated amounts of retinyl acetate in corn oil were administered from weaning until 7 days after treatment with DMBA. Thereafter all rats were given retinyl acetate, 10 µg/day, until termination. Ages of rats at termination for Experiments 1 to 3 were 173, 290, and 272 days, respectively.

³ DMBA was injected in the tail vein.

⁴ Unlike letters (w, x, y, z) denote significance, P ≤ 0.05.

⁵ Numbers in parentheses, number of animals in each group.

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Table 2 Effect of retinyl acetate administration during initiation on the number of mammary carcinomas and mammary fibroadenomas in female rats treated with low dose levels of DMBA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Carcinomas</th>
<th>Fibroadenomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMBA (mg/100 g body wt)*</td>
<td>Retinyl acetate (µg/day)*</td>
<td>Final body wt (g)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A, marginal vitamin A</td>
<td>0.5</td>
<td>3</td>
</tr>
<tr>
<td>B, adequate vitamin A</td>
<td>0.5</td>
<td>10</td>
</tr>
<tr>
<td>C, moderately increased vitamin A</td>
<td>0.5</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A, marginal vitamin A</td>
<td>0.1</td>
<td>3</td>
</tr>
<tr>
<td>B, adequate vitamin A</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>C, moderately increased vitamin A</td>
<td>0.1</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A, marginal vitamin A</td>
<td>0.1</td>
<td>3</td>
</tr>
<tr>
<td>B, adequate vitamin A</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>C, moderately increased vitamin A</td>
<td>0.1</td>
<td>30</td>
</tr>
</tbody>
</table>

* DMBA was injected in the tail vein.

† The indicated amounts of retinyl acetate in corn oil were administered from weaning until 7 days after treatment with DMBA. Thereafter all rats were given 10 µg retinyl acetate until termination. Ages of rats at termination for Experiments 1 to 3 were 173, 290, and 272 days, respectively.

‡ Mean ± SE.

§ Unlike letters (w, x, y) denote significance, P < 0.05.

Table 3 Effect of retinyl acetate administration during promotion on total number of mammary tumors in female rats treated with low dose levels of DMBA

<table>
<thead>
<tr>
<th>Total no. of mammary tumors</th>
<th>DMBA (mg/100 g body wt)*</th>
<th>Combined experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>Group</td>
<td>Experiment 1</td>
<td>Experiment 2</td>
</tr>
<tr>
<td>A, marginal vitamin A</td>
<td>3</td>
<td>110x (55)x</td>
</tr>
<tr>
<td>B, adequate vitamin A</td>
<td>10</td>
<td>107x (53)</td>
</tr>
<tr>
<td>C, moderately increased vitamin A</td>
<td>30</td>
<td>118x (55)</td>
</tr>
</tbody>
</table>

* The indicated amounts of retinyl acetate in corn oil were administered from 7 days after treatment with DMBA until termination of experiment; prior to that time and since weaning, all rats were given retinyl acetate, 10 µg/day. Ages of rats at termination for Experiments 1 to 3 were 173, 290, and 272 days, respectively.

† DMBA was injected in the tail vein.

‡ Unlike letters (w, x, y, z) denote significance, P < 0.05.

§ Numbers in parentheses, number of animals in each group.

ing marginal levels of vitamin A; these differences in mammary carcinoma number were not significant. In contrast, the total numbers of mammary fibroadenomas (combining Experiments 2 and 3) were reduced by 42 and 58% in rats fed moderately increased and marginal amounts, respectively, of vitamin A; this reduction was significant. The percentage of changes in mammary tumor number and statistical analyses were adjusted to account for group size differences. The mean percentage of rats bearing mammary carcinomas in the groups of rats fed 3, 10, and 30 µg of vitamin A per day was 37, 44, and 40%, respectively (combining Experiments 1 to 3); the mean percentage of rats bearing benign mammary fibroadenomas was 40, 45, and 49%, respectively (combining Experiments 2 and 3) (Table 4). The percentage of rats bearing either benign or carcinomatous mammary tumors was not significantly altered by the varied dietary intake of vitamin A during the promotion stage of mammary tumorigenesis.

Effect of Dietary Lack of Retinyl Acetate on the Initiation and Promotion Stages of Low Dose Level DMBA-induced Rat Mammary Tumorigenesis. In female rats fed a diet without vitamin A (0 µg/day) prior to and during the administration of a low dose level of DMBA (initiation study), 20 mammary carcinomas were observed in 51 rats, a frequency of carcinomas that did not differ significantly from the group of rats receiving adequate levels of vitamin A (10 µg/day) and that were subjected to restricted food consumption, so as to equalize body weight gains (17 mammary carcinomas in 48 rats) (Table 5). Mammary fibroadenoma frequency was also not significantly different in rats fed a diet without vitamin A (67 mammary fibroadenomas in 51 rats), when compared to rats receiving adequate vitamin A and food restricted (58 mammary fibroadenomas in 48 rats). Similarly, the percentage of rats bearing mammary carcinomas or mammary fibroadenomas was not significantly different in the two groups of rats.

In female rats fed a diet without vitamin A (0 µg/day) commencing 7 days after the administration of a low dose level of DMBA (promotion study), 6 mammary carcinomas and 12 mammary fibroadenomas were observed in 49 rats; 5 mammary carcinomas and 17 mammary fibroadenomas were observed in an equal number of rats receiving adequate vitamin A (10 µg/day) and food restricted (during the last 2 mo of the postcarcinogen period) to equalize body weight gains (Table 5). These differences in numbers of mammary carcinomas and mammary fibroadenomas in the two groups of rats were not statistically significant. Similarly, the percentage of rats bearing mammary carcinomas or mammary fibroadenomas was not significantly different in the two groups of rats.

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Table 4 Effect of retinyl acetate administration during promotion on the incidence of mammary carcinomas and mammary fibroadenomas in female rats treated with low dose levels of DMBA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mammary tumors</th>
<th>Carcinomas</th>
<th>Fibroadenomas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMBA (mg/100 g body wt)</td>
<td>Retinyl acetate (µg/day)</td>
<td>Final body wt (g)</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------------</td>
<td>------------</td>
<td>--------------</td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A, marginal vitamin A</td>
<td>0.5</td>
<td>3</td>
<td>333 ± 5x</td>
</tr>
<tr>
<td>B, adequate vitamin A</td>
<td>0.5</td>
<td>10</td>
<td>336 ± 5x</td>
</tr>
<tr>
<td>C, moderately increased vitamin A</td>
<td>0.5</td>
<td>30</td>
<td>335 ± 5x</td>
</tr>
</tbody>
</table>

**Experiment 2**

| A, marginal vitamin A | 0.1 | 3 | 387 ± 8x | 17x | 11/48 | 23x | 0.4w | 33x | 14/48 | 29w | 0.7w |
| B, adequate vitamin A | 0.1 | 10 | 376 ± 5x | 30 | 16/47 | 34x | 0.6x | 49x | 15/47 | 32w | 1.0w |
| C, moderately increased vitamin A | 0.1 | 30 | 380 ± 7x | 15x | 11/49 | 22x | 0.3w | 37x | 15/49 | 31w | 0.8w |

**Experiment 3**

| A, marginal vitamin A | 0.1 | 3 | 350 ± 7x | 13w | 11/50 | 22w | 0.3w | 93x | 25/50 | 50w | 1.9w |
| B, adequate vitamin A | 0.1 | 10 | 358 ± 5x | 23w | 15/50 | 30w | 0.5w | 248x | 29/50 | 58w | 5.0x |
| C, moderately increased vitamin A | 0.1 | 30 | 358 ± 8x | 33x | 15/50 | 30w | 0.7x | 137y | 33/50 | 66y | 2.7w |

* DMBA was injected in the tail vein.  
† The indicated amounts of retinyl acetate in corn oil were administered from 7 days after treatment with DMBA until termination of experiment; prior to that time and since weaning, all rats were given retinyl acetate, 10 µg/day. Ages of rats at termination for Experiments 1 to 3 were 173, 290, and 272 days, respectively.  
‡ Mean ± SE.  
§ Unlike letters (w, x) denote significance, P ≤ 0.05.

Table 5 Effect of feeding a diet lacking in vitamin A (retinyl acetate) on the number of mammary carcinomas and mammary fibroadenomas in female rats treated with low dose levels of DMBA, 0.1 mg/100 g body weight, injected into the tail vein

<table>
<thead>
<tr>
<th>Groups</th>
<th>Carcinomas</th>
<th>Fibroadenomas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of rats</td>
<td>Total no. of tumors</td>
</tr>
<tr>
<td>D, lack of vitamin A</td>
<td>0</td>
<td>363 ± 6w</td>
</tr>
<tr>
<td>E, adequate vitamin A</td>
<td>10w</td>
<td>357 ± 8w</td>
</tr>
<tr>
<td>Promotion study†</td>
<td>D, lack of vitamin A</td>
<td>0</td>
</tr>
<tr>
<td>E, adequate vitamin A</td>
<td>10w</td>
<td>337 ± 4w</td>
</tr>
</tbody>
</table>

* The indicated amounts of retinyl acetate in corn oil were administered from 7 days after treatment with DMBA until termination of experiment. Thereafter all rats were given retinyl acetate, 10 µg/day, until termination. Age of rats at termination was 290 days.  
† Mean ± SE.  
‡ Unlike letters denote significance, P ≤ 0.05.  
§ Feed intake restricted to maintain a mean body weight within 10 g of that of rats in Group D.

that received an adequate amount of vitamin A (20 µg/day) and were fed ad libitum (Experiment 2; Tables 2, 4, and 5).

Liver and Plasma Vitamin A Values in Female Rats Fed Graded Daily Amounts of Retinyl Acetate. Vitamin A values were assessed in all groups of rats in Experiment 2. The mean liver vitamin A of the weanling female rats at the beginning of the experiment was 26.5 µg/g; they had 11.0 µg of retinol per dl of plasma (Table 6). The mean liver vitamin A 24 h prior to DMBA administration (initiation study) (56 days of retinyl acetate supplementation) was 41.9 µg/g in rats fed an adequate amount of retinyl acetate (10 µg/day; controls). Significantly less (3.9 µg/g) vitamin A was found in the livers of rats fed diets lacking vitamin A (0 µg/day) or those fed marginal amounts of retinyl acetate (3 µg/day) (2.3 µg/g); rats fed a diet with moderately increased amounts of retinyl acetate (30 µg/day) had significantly increased levels (165.8 µg/g). Vitamin A in their livers. The mean plasma retinol values (9.6 to 12.2 µg/dl) were not significantly different among the rats supplemented with 3 and 10 µg of retinyl acetate. However, plasma retinol was slightly increased (15.7 µg/dl) in the group of rats maintained on moderately increased retinyl acetate supplementation (30 µg/day); this increase just reached the 5% level of statistical significance. Rats fed diets lacking retinyl acetate (0 µg/day) had significantly lower plasma retinol, as compared to rats given retinyl acetate. In the promotion study, the liver vitamin A value at termination (28 days of retinyl acetate supplementation) was 72.0 µg/g in rats fed an adequate amount of retinyl acetate (10 µg/day, controls). Significantly less vitamin A was found in livers of rats fed diets lacking or marginally supplemented with retinyl acetate (0 or 3 µg/day, respectively); rats fed a moderately increased supplement of retinyl acetate (30 µg/day) had significantly increased liver vitamin A. Mean plasma retinol values (9.2 to 14.0 µg/dl) were not significantly different among the retinyl acetate-supplemented groups (3, 10, and 30 µg/day), while rats fed diets lacking retinyl acetate (0 µg/day) had negligible amounts of plasma retinol.

Effect of Retinyl Acetate Feeding on Body Weight Gains. A representative illustration of growth characteristics of rats in our studies is provided in Fig. 1 (Experiment 2, initiation study) and in Fig. 2 (Experiment 2, promotion study). In both the
Table 6. Plasma and liver vitamin A values in female Sprague-Dawley rats fed specified daily amounts of retinyl acetate during the initiation and promotional stages of DMBA-induced mammary gland carcinogenesis.

<table>
<thead>
<tr>
<th>Retinyl acetate (μg/day)</th>
<th>Retinyl acetate treatment (days)</th>
<th>Age of rats (days)</th>
<th>Liver vitamin A (μg/g)</th>
<th>Plasma retinol (μg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initiation study*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>44</td>
<td>20</td>
<td>26.4 ± 4.0f</td>
<td>(6)* 11.0 ± 3.0</td>
</tr>
<tr>
<td>3</td>
<td>44</td>
<td>63</td>
<td>3.9 ± 3.2w*</td>
<td>(6) 1.5 ± 2.0w</td>
</tr>
<tr>
<td>10</td>
<td>44</td>
<td>64</td>
<td>2.3 ± 4.7w</td>
<td>(6) 9.6 ± 1.4x</td>
</tr>
<tr>
<td>30</td>
<td>44</td>
<td>64</td>
<td>41.9 ± 18.0x</td>
<td>(6) 12.1 ± 4.4x</td>
</tr>
<tr>
<td>10*</td>
<td>44</td>
<td>64</td>
<td>165.8 ± 45.1y</td>
<td>(5) 15.7 ± 2.4y</td>
</tr>
<tr>
<td>Promotion study*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>218</td>
<td>290</td>
<td>37.1 ± 13.6w</td>
<td>(6) 0.8 ± 1.2w</td>
</tr>
<tr>
<td>3</td>
<td>218</td>
<td>290</td>
<td>38.0 ± 12.0w</td>
<td>(5) 9.2 ± 2.7x</td>
</tr>
<tr>
<td>10</td>
<td>218</td>
<td>290</td>
<td>72.0 ± 17.7x</td>
<td>(6) 11.5 ± 5.5x</td>
</tr>
<tr>
<td>30</td>
<td>218</td>
<td>290</td>
<td>476.8 ± 54.8y</td>
<td>(6) 14.0 ± 5.6x</td>
</tr>
<tr>
<td>10</td>
<td>218</td>
<td>290</td>
<td>73.1 ± 13.2x</td>
<td>(6) 10.7 ± 2.1x</td>
</tr>
</tbody>
</table>

a Retinyl esters and retinol, combined values.

b Rats received specified retinyl acetate treatments from time of weaning (20 days) until the age of 64 days, at which time liver biopsies and blood samples were taken for analysis, and the rats were returned to the study. DMBA treatment was at 65 days of age.

c Mean ± SD.

d Numbers in parentheses, number of rats.

* Unlike letters (w, x, y) denote significance, P ≤ 0.05.

Table 6 Plasma and liver vitamin A values in female Sprague-Dawley rats fed specified daily amounts of retinyl acetate during the initiation and promotional stages of DMBA-induced mammary gland carcinogenesis.

All data in this table are from Experiment 2.

Discussion

Epidemiological studies, while not conclusive, offer compelling evidence that an ample dietary consumption of vitamin A-containing foods reduces the risk of many epithelial cancers, including breast cancer (12, 13, 22, 39, 40). These findings are in accord with experimental data based on numerous animal models where the development of chemically induced epithelial tumors has been successfully inhibited or prevented with pharmacological levels of natural and synthetic retinoids (11, 19, 44, 45). Retinyl acetate has been especially effective in suppressing chemically induced mammary neoplasms in rodent models; however, in these experiments only pharmacological amounts of retinyl acetate have been examined (19–24). There have been no studies on the incidence of induced mammary tumors in animals lacking dietary vitamin A or concerning the possible beneficial effects of moderate but sustained vitamin A ingestion for the prevention or lowering of mammary tumor incidence. Such studies are necessary in order to interpret the epidemiological data, since the nutritional information that is available from epidemiological studies does not provide an accurate quantitative estimate of vitamin A consumption, nor does it exclude other nutrient categories, associated with vitamin A or provitamin A (carotenes) consumption, that themselves might have anticarcinogenic effects (12, 13).

Epidemiological evidence from many prospective and retrospective serological studies also points to a link between lower than average circulatory amounts of vitamin A and an increased risk of many types of epithelial cancer (12, 13, 43). However, there have been no studies that correlate the amount of circulatory vitamin A with the effective chemopreventive level of administered retinyl acetate at critical periods in carcinogenesis or at time of tumor growth suppression. Serological data in humans suggest that the lower the serum vitamin A (even though within the normal range), the higher the risk of epithelial cancers (12). These observations necessitate confirmation in experimental animal carcinogenesis models, particularly...
since recent serological studies have yielded conflicting results (44–47).

The development of reliable experimental animal models for benign breast disease has been largely ignored. Benign breast disease, although lacking the lethality (and thus, the thrust of research) of the malignant disease, remains a common and nagging problem in our population as the incidence is as high as 89% in United States women (48). Few studies have been conducted to examine the effect of vitamin A nutrition on the incidence of benign mammary tumors in experimental animals.

In the studies reported here we have addressed several important aspects in the relationship of vitamin A nutrition and mammary tumor development. We examined mammary tumor incidence and the course of tumor development using very low doses of DMBA. This experimental design was necessary so as to produce a frequency of benign and carcinomatous mammary gland tumors that would be more akin to that occurring in human female populations (49). Furthermore, a low carcinogen dose more closely approaches the human environmental exposure to carcinogens. Most importantly, the study was designed to examine the relationship of relatively normal vitamin A nutritional status to mammary carcinogenesis, so as to ultimately provide safe, nutritional guidelines for tumor prophylaxis, compatible with human dietary practices.

The use of a very low dose level of DMBA is not, however, without problems. The frequency of mammary tumors, especially mammary carcinomas, is of such low magnitude, that extremely large numbers of experimental animals are needed to generate sufficient tumor numbers for statistical analysis. This necessitates repeating identical (or near identical) experiments and, upon termination of experiments, the combining of results of all groups from each experiment which received identical treatments. In the present study, we combined Experiments 1 to 3 and Experiments 2 and 3 in order to generate sufficient numbers of mammary tumors for a meaningful statistical evaluation; such experimental combinations resulted in approximately 100 animals (Experiments 2 and 3, combined) or 150 animals (Experiments 1 to 3, combined) per experimental group. In these studies, the only interexperimental variables were: the time of year in which these experiments were performed (during a 3-yr period); the duration of experiment (Experiment 1, 173 days, and Experiments 2 and 3, 290 and 272 days); and the amount and time of dose of the carcinogen (Experiment 1, 0.5 mg DMBA, 63d, and Experiments 2 and 3, 0.1 mg DMBA, 65d and 55d). Despite such efforts, mammary carcinoma frequency, unlike that of the benign mammary tumors, was too low (Experiments 2 and 3) to generate significant differences among treatment groups. Nevertheless, a number of definitive statistically supported conclusions have been obtained from this study. Our salient conclusions are as follows.

(a) A prophylactic effect against rat mammary tumorigenesis has been demonstrated during a sustained consumption of amounts of vitamin A that are within the normal dietary range of the vitamin and in the absence of influences of other dietary variables.

(b) Moderate dietary supplementation of vitamin A (only 3 times the adequate amount), when provided during the initiation or promotion stage of low dose DMBA-induced rat mammary gland tumorigenesis, significantly \((P < 0.05)\) reduced the occurrence of mammary tumors; such diets did not affect body weight gains.

(c) Marginal ingestion of vitamin A (sufficient to maintain good health and normal plasma retinol levels, but insufficient to result in significant liver reserves of the vitamin), when provided during the initiation or promotion stage of low dose DMBA-induced rat mammary gland tumorigenesis, also significantly \((P < 0.05)\) reduced the occurrence of mammary tumors; such diets did not affect body weight gains.

(d) Diets lacking in vitamin A, when provided during the initiation or promotion stage of low dose DMBA-induced rat mammary gland tumorigenesis, significantly \((P < 0.05)\) reduced the occurrence of mammary tumors; such diets resulted in reduced body weight gains. However, the incidence of mammary tumor in these rats was similar to that of rats fed adequate vitamin A but subjected to restricted food intake to equalize body weight gains.

(e) The profound inhibitory effect of moderately increased vitamin A supplementation or marginally low dietary levels of the vitamin on this carcinogenic process was reflected primarily in significant \((P < 0.05)\) reductions in benign mammary fibroadenomas; mammary carcinoma numbers were often reduced during these treatments, but the reductions did not reach the 5% level of statistical probability.

Our finding, that only a moderately increased frequent ingestion of retinyl acetate during initiation and promotion was sufficient to lower the subsequent mammary tumor occurrence, is in accord with the earlier observations of Nettesheim and Williams (9), who demonstrated that a moderate daily intake of retinyl acetate was effective in the prevention of development of 3-methylcholanthrene-induced neoplastic lung nodules and that no additional protection resulted using pharmacological amounts of retinoids in this lung carcinogenesis model. Furthermore, the availability of considerable stores of vitamin A in the liver was ineffective in the prevention of these neoplasms, if dietary vitamin A was not available. Our observations and those of Nettesheim and Williams thus illustrate the importance of a continued dietary availability of vitamin A for the prevention of neoplastic development in at least two epithelial tissues: the mammary gland and the lung. It is possible that other epithelial carcinogenesis models will respond in a similar manner.

Studies on vitamin A metabolism (reviewed in Ref. 50) suggest that there are several body pools of vitamin A, and that newly absorbed vitamin A is in a separate compartment from that of previously stored vitamin A; there is no mixing of these compartments for at least 24 h. About 80% of circulating retinol is derived from this newly absorbed vitamin A. Thus, it appears that the circulatory retinol derived from the new, nonequilibrated vitamin A pool is responsible for the antitumorigenic effects that we observed in our studies. It is also possible that the amount of vitamin A ingested may not be as important as long as there is a continued supply of vitamin A to this compartment. This concept is supported by experimental work with pharmacological doses of retinyl acetate: withholding the feeding of 1 mM retinyl acetate in the diet during a period of effective mammary tumor suppression resulted in a rapid appearance of tumors (21, 23, 24, 51–53), although the livers of these rats should have had a considerable amount of vitamin A. We have determined that livers of rats treated for 8 wk with 1.0 mM retinyl acetate in the diet contain about 2.7 mg of vitamin A esters per g of liver. Livers of rats fed the above diet for several months would accumulate even higher amounts of vitamin A. Thus, it appears that vitamin A stores in the liver are not directly related to reduced mammary tumor incidence, since the presumed presence of considerable vitamin A stores in the liver was ineffective in tumor suppression in the absence of a continued dietary availability of vitamin A.

4 Unpublished results.
of continuous dietary vitamin A (21, 23, 24, 51–53).

Epidemiological studies (12, 13, 39) have linked the ingestion of low dietary vitamin A to an increased risk of breast disease. However, these studies did not explore the possible association of low dietary vitamin A ingestion with decreased circulatory vitamin A. Similarly, the serological studies that link breast cancer with lower than normal circulatory vitamin A levels (12, 54, 55) have not been matched with corresponding dietary histories. In our studies reported here, for the first time in an experimental animal tumorigenesis model, an attempt was made to correlate retinyl acetate ingestion, plasma vitamin A levels, and subsequent tumor development. A low amount of circulatory retinol (characteristic of the vitamin A-depleted state) during mammary tumor initiation (Experiment 2, initiation study) was not associated with an increased occurrence of mammary tumors. Similarly, low serum retinol levels by the end of the promotion phase (Experiment 2, promotion study) did not significantly increase the development of mammary tumors. Our data suggest that decreased circulatory levels of retinol are not directly linked to an increased risk of mammary tumors.

Our observations, in addition, suggest that mammary tumorigenesis is not significantly influenced by the vitamin A-deficient state, in contrast to the reported enhanced susceptibility to cancer induction in epithelia such as the gastrointestinal tract, urinary tract, respiratory tract, and oral mucosa of the vitamin A-depleted animal (3–11). The different response observed in the mammary gland may be due to the role of vitamin A in regulating the normal development of the mammary gland; i.e., vitamin A-deficient rodents appear to have an underdeveloped mammary gland (56). It is well known that the hypoplastic rat mammary gland is considerably less susceptible to tumor initiation by DMBA than is the normally developed gland (57).

A profoundly important observation in our study is that a moderately (3-fold) increased consumption of vitamin A prior to and during the initiation of low dose DMBA-induced mammary tumorigenesis decreased the occurrence of mammary tumors. This dietary vitamin A treatment resulted in a 30% increase in serum retinol at tumor initiation, an increase that was statistically significant. Kummet et al. (12) suggest that a sustained ingestion of increased amounts of vitamin A can alter, although only slightly, circulatory vitamin A levels, but that this elevation of serum retinol may be responsible for the prophylactic effect of dietary vitamin A against many epithelial cancers. One of the physiological functions of vitamin A is the maintenance of defense mechanisms, such as those involved in the modification of carcinogen metabolism and the repair of DNA (21, 58); it is conceivable that an enhancement of these mechanisms by vitamin A at tumor initiation was responsible for the subsequent decrease in mammary tumor development observed in our studies.

The decreased occurrence of mammary tumors in rats consuming marginal amounts of vitamin A, however, cannot be explained by an increased amount of plasma retinol at the time of tumor initiation, since in these rats plasma retinol levels were not significantly different from those of adequately supplemented rats. The data suggest that marginal ingestion of vitamin A, when accompanied by normal body growth and normal plasma levels of retinol, is a deterrent to mammary tumor development. A possible explanation may be that marginal vitamin A consumption, while adequate for many physiological functions in tissues, may not provide a nutritionally adequate environment for neoplastic cell development. This interpretation is based on the general observation that an inadequacy of any nutrient most often will retard the carcinogenic process.

The well-known influence of nutritional deprivation on the carcinogenesis process (59) was also evident in our study. It remains to be determined whether the observed general decrease in overall tumor development is due to restriction of specific nutrient ingestion or to a limitation of caloric consumption.

Our experimental design made it possible to delineate at what stage of mammary carcinogenesis vitamin A is effective; we observed an inhibition of mammary tumor development during initiation as well as during the promotion stage. McCormick et al. (52) obtained similar results with pharmacological amounts of retinyl acetate in the DMBA-induced rat mammary tumor model. Others (51, 60), however, have concluded that retinyl acetate inhibition of carcinogen-induced rat mammary tumorigenesis was related primarily to an effect on the promotion stage of this tumorigenic process. In rodent skin carcinogenesis models, retinoids are effective only during the promotion stage (61, 62); in vitro studies also indicate that retinoids act as antipromoters (63–65). Possible anticarcinogenic mechanisms of action of retinoids during the promotion stage include inhibition of tumor cell proliferation, altered tumor cell differentiation, enhanced antitumor immune responses, and suppression of transforming growth factors (21, 61–67).

Our data clearly point out the complex nature of the in vivo anticarcinogenic action of vitamin A. From our studies we conclude that moderate alterations in daily vitamin A consumption can indeed modulate mammary gland tumorigenesis, at both the initiation and promotion stages; most importantly, suppression of low dose chemically induced mammary tumorigenesis can be achieved by moderately increased, sustained consumption of vitamin A. It is hoped that the results of the studies reported here will provide a basis for the search of a nutritionally sound and medically safe prophylactic approach for the use of vitamin A in the control of mammary tumorigenesis.

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EFFECT OF VITAMIN A ON THE DEVELOPMENT OF RAT MAMMARY TUMORS


Effect of Moderate Vitamin A Supplementation and Lack of Dietary Vitamin A on the Development of Mammary Tumors in Female Rats Treated with Low Carcinogenic Dose Levels of 7,12-Dimethylbenz(a)anthracene


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