

# Effects of Dietary Retinyl Palmitate or 13-*cis*-Retinoic Acid on the Promotion of Tumors in Mouse Skin<sup>1</sup>

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## ABSTRACT

The present study was designed to determine the effects of dietary 13-*cis*-retinoic acid and retinyl palmitate on mouse skin tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Female CD-1 mice were initiated with 150 nmol of 7,12-dimethylbenz(a)anthracene and promoted twice weekly with 8 nmol of TPA. Diets supplemented with retinyl palmitate to yield 60,000 or 200,000 IU or 700,000 for 5 wk followed by 350,000 IU per kg of diet (700,000/350,000) fed to mice during tumor promotion resulted in 9%, 37%, and 65% inhibition of the papilloma yield, respectively, at 21 wk of promotion. Although topical applications of 13-*cis*-retinoic acid have been almost as effective as retinoic acid in preventing the appearance of mouse skin tumors, dietary 13-*cis*-retinoic acid at 200,000 or 700,000 IU per kg of diet resulted in no reduction in papilloma yield but did result in a dose-dependent decrease in the tumor burden (weight of tumors per mouse). Therefore, dietary retinyl palmitate yielded a dose-dependent inhibition of the number and weight of tumors promoted by TPA, whereas dietary 13-*cis*-retinoic acid resulted in a decrease in weight but not in number of tumors promoted by TPA.

## INTRODUCTION

Continuous administration of retinoids has been shown to prevent cancer of the skin (1-3), lung (4), bladder (5), and breast (6) in experimental animals exposed to carcinogens. The mechanism for this preventive action of retinoids is not known. Although most of the studies concerning chemoprevention have been conducted on animal model systems, epidemiological results suggest that dietary retinoids may be chemopreventive to some forms of cancer in humans as well (7-10).

The toxicity of vitamin A precludes its sustained use as a form of cancer prevention. Therefore it is important to determine which vitamin A analogues lead to increased levels of chemoprevention without concomitant increased levels of toxicity. Chemoprevention of mouse skin tumorigenesis by retinyl palmitate has not been reported previously. To determine their relative chemopreventive capacity, we have measured the effect of selected doses of dietary retinyl palmitate and 13-*cis*-retinoic acid on mouse skin papilloma promotion by TPA.<sup>4</sup> The anti-promotion activity was specifically determined, because retinoids are selectively active during the promotion phase of mouse skin tumorigenesis (1, 11), and tumor prevention at intermediate stages of carcinogenesis is of potential clinical significance.

## MATERIALS AND METHODS

**Materials.** Female CD-1 mice were purchased from the Charles River Breeding Laboratory, Wilmington, MA, and were used for experimen-

Received 5/12/86; revised 11/3/86; accepted 11/7/86.

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<sup>1</sup> Supported by NIH Grant CA-27502, Phi Beta Psi Sorority, and Wallace Genetics, Inc.

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<sup>4</sup> The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; RP, retinyl palmitate; CRA, 13-*cis*-retinoic acid; DMBA, 7,12-dimethylbenz(a)-anthracene.

tation at 6 wk of age. TPA was obtained from Chemical Carcinogenesis, Eden Prairie, MN. DMBA was purchased from Sigma Chemical Company, St. Louis, MO. Retinoids were a gift from Hoffmann-La Roche, Inc., Nutley, NJ.

**Treatment of Mice.** All mice were housed in hanging cages in a light- and temperature-controlled room isolated from other animals. Food and water were available *ad libitum*. The dorsal skin of the mice was shaved 2 days before initiation, and only those mice in the resting phase of the hair cycle were used for experimentation. The solutions of TPA and DMBA were prepared in spectrograde acetone and applied to the shaved backs of individual mice in a volume of 0.2 ml. Control mice were treated with the same volume of acetone.

**Tumor Induction.** Tumors were induced by the initiation-promotion regimen (1). Initiation consisted of a single application of 150 nmol of DMBA in 0.2 ml of acetone; 3 wk later, TPA (8 nmol) was applied in 0.2 ml of acetone twice weekly for the duration of the experiment. Papillomas were counted weekly.

**Diet.** Basal diet for the study was the American Institute of Nutrition semipurified rat-mouse powdered Diet 76A (US Biochemical). The retinyl palmitate level in the basal diet was reported by the manufacturer as 3900 IU/kg of diet, as added RP. This was confirmed by high-performance liquid chromatography, assayed by Dr. Peng in Dr. Albert's laboratory, Department of Pharmacology, University of Arizona. Retinoid-supplemented diets contained additional amounts of the test compound, as shown in Table 1. CRA levels were also measured by Dr. Peng. To prepare experimental diets, synthetic RP in powder form, type 250-SD, or 13-*cis*-retinoic acid (at a concentration of 11.5% in beadlets) was mixed with the basal powdered diet at intervals of 2 wk. The biopotency of 13-*cis*-retinoic acid is 1 IU/0.31  $\mu$ g, so that 200,000 IU represents 62 mg. The diets were stored at -4°C and fed to the mice daily in a room with no direct lighting.

All mice received the basal diet and distilled water for 2 wk before and 3 wk after initiation with DMBA. Retinoid treatments were concurrent with TPA promotion in order to study specifically the dietary influences on promotion. Mice were randomly divided into groups of 35 mice each, as shown in Table 1, and all groups were studied concurrently so that results could be compared. Ten mice from each group were weighed every other week. Food consumption was measured periodically, as shown in Figs. 3 and 4.

## RESULTS

**Inhibition by Dietary Retinyl Palmitate of Skin Tumors Promoted by TPA.** Dietary retinyl palmitate fed during promotion with TPA to DMBA-initiated mice inhibited the formation of skin papillomas in a dose-dependent fashion, as shown in Fig. 1. Retinyl palmitate at concentrations of 60, 200, or 350 IU/g of diet inhibited the number of papillomas/mouse by 13, 32, and 48%, respectively. Analysis of variance was performed on the number of papillomas/mouse. The results at 16 wk of promotion were analyzed to represent the preventive activity of dietary retinyl palmitate. At this time there was a significant difference between the groups treated with 350,000 IU of retinyl palmitate and the control group ( $P = 0.006$ ). The difference between the group treated with 200,000 IU of RP and the control group verged on significance ( $P = 0.06$ ). The values at 23 wk of promotion were considered to represent both preventive and therapeutic activity of dietary retinyl palmitate, because regression as well as prevention was evident at this time. The

numbers of papillomas/mouse in groups which received 200,000 or 350,000 IU of retinyl palmitate were significantly different from the value of the control group ( $P < 0.02$ ). The rate at which mice became tumor bearers and the final number of papilloma-bearing mice were similar in control mice and in mice fed 60,000 to 200,000 IU of RP per kg of food, but was reduced ( $P < 0.05$ ) in mice fed 350,000 IU of RP per kg of food (Fig. 1).

**Influence of 13-*cis*-Retinoic Acid on Mouse Papilloma Promotion by TPA.** Mice fed with a diet supplemented with 200,000 or 700,000 IU of CRA did not have a significantly different number of TPA-promoted tumors than did control mice, based on a 95% confidence level in the Student *t* test (Fig. 2). There was also no difference between CRA-treated and control mice in the rate at which mice became tumor bearers or in the final proportion of tumor bearers. However, CRA treatment resulted in a dose-dependent inhibition of the tumor burden per mouse, as measured by the weight of the tumors per mouse (Table 2). A dose-dependent inhibition of tumor burden was also found with dietary retinyl palmitate. The extent of inhibition was significant at all levels of retinoid supplementation beyond the basal diet component, based on the 95% confidence level of the Student *t* test.

**Effect of Retinoids on Body Weight.** Since caloric restriction can inhibit tumor promotion (12), mean food consumption and mean body weights during the promotion phase were determined, and the data are presented in Figs. 3 and 4. There was no statistically significant difference in food consumption between treated and control mice; however, there was a 16% difference in body weight between control mice and those fed at the highest concentration of RP. This influence may play a contributing role in the antitumor activity at the highest concentration of retinyl palmitate. However, this effect was titrated out at a dose level that still had a dramatic inhibitory effect on tumor promotion as assayed by tumor burden per mouse.

**Toxicity of Retinyl Palmitate.** Although mice fed with 700,000 IU of CRA per kg of diet appeared healthy throughout the study, mice fed with 700,000 IU of retinyl palmitate per kg of food displayed symptoms of toxicity: reduced body weight gain (insignificant by the Student *t* test); edema noted in the ears and around the eyes; bone deformities; and death. By the sixth wk of tumor promotion, 12 of 70 mice treated with the highest dose of RP had died from retinoid toxicity. At the fifth wk, the diet was adjusted so that this group of mice received 1/2 the dose it had been receiving, as is practiced in human clinics studying retinoid chemoprevention. With this diet adjustment, the

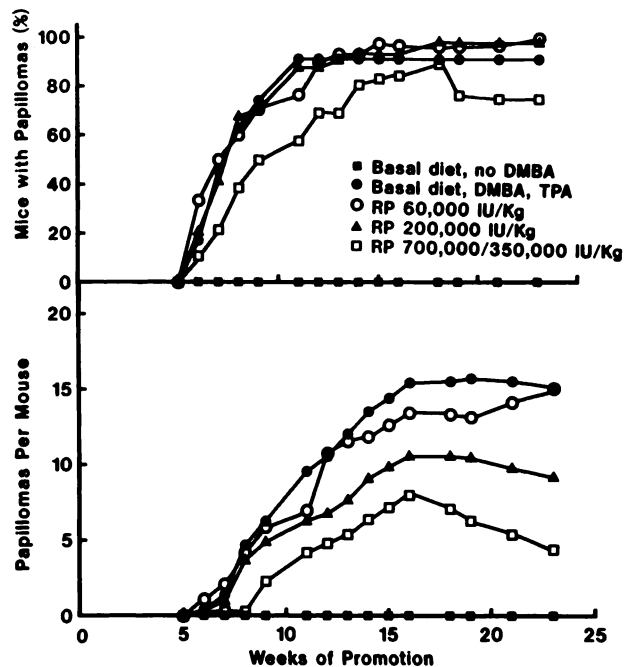


Fig. 1. The effect of dietary retinyl palmitate on TPA-promoted mouse skin papilloma formation. Mice were treated as described in Table 1.

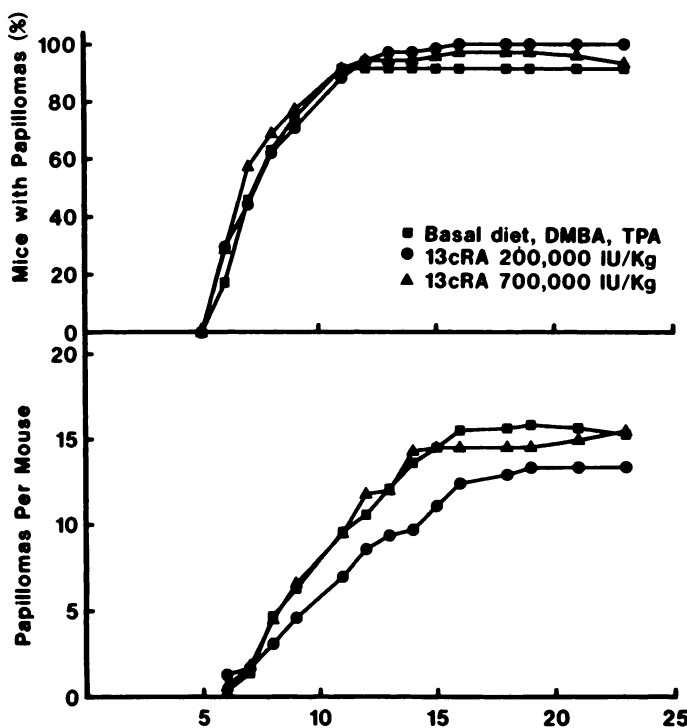


Fig. 2. The effects of dietary 13-*cis*-retinoic acid on TPA-promoted mouse skin papilloma formation. Mice were treated as described in Table 1.

Table 1 Experimental design of dietary treatment of mice during TPA promotion of initiated mice

All mice received the basal diet for 2 wk before and 3 wk after initiation with 0.15  $\mu$ mol of DMBA. At that time, TPA promotion and dietary treatment began and continued for 21 wk. Each dietary group consisted of 35 mice.

Group	Basal diet <sup>a</sup>	DMBA <sup>b</sup>	TPA <sup>c</sup>	RP (IU/kg) <sup>d</sup>	Supplementary CRA (IU/kg) <sup>e</sup>
Basal diet	+	-	-	-	-
Control	+	+	+	-	-
60 RP	+	+	+	60,000	-
200 RP	+	+	+	200,000	-
700 RP	+	+	+	700,000 for 5 wk, 350,000 thereafter	-
200 CRA	+	+	+	-	200,000
700 CRA	+	+	+	-	700,000

<sup>a</sup> The basal diet contained 3,900 IU of RP per kg.

<sup>b</sup> DMBA, 0.15  $\mu$ mol in 0.2 ml of acetone.

<sup>c</sup> TPA, 8 nmol in 0.2 ml of acetone, twice weekly.

<sup>d</sup> Biopotency of retinyl palmitate: 1 IU/0.55  $\mu$ g; therefore 60,000 IU = 33 mg.

<sup>e</sup> CRA, 1 IU/0.31  $\mu$ g; therefore 200,000 IU = 62 mg.

deaths ceased. Thus, retinyl palmitate was significantly more toxic than was 13-*cis*-retinoic acid, at equal dose levels in the diet.

DISCUSSION

There was a dose response in CD-1 mice for the inhibition of TPA-induced mouse skin tumor promotion by dietary retinyl palmitate. Mice fed with a diet supplemented with retinyl palmitate to a level of 60,000 or 200,000 IU or 700,000 IU for 5 wk and then 350,000 IU per kg of diet demonstrated 9%,

Table 2 Effect of retinyl palmitate or 13-cis-retinoic acid on mouse skin tumor burden

All mice were initiated and promoted as described in "Materials and Methods." The basal diet contained 3,900 IU of retinyl palmitate per kg; this diet was supplemented in the test mice to yield the indicated amounts. Tumors from 15 mice of each group were removed and weighed at 21 wk of promotion.

Treatment	Supplementation (IU/kg diet)	Tumors/mouse	Wt of tumors (g)/mouse
Basal diet	3,900	15.6 ± 2.7 <sup>a</sup>	1.373 ± 0.20 <sup>a</sup>
RP	60,000	14.3 ± 2.6	0.20 ± 0.04
	200,000	8.2 ± 2.0	0.131 ± 0.03
	700,000/350,000	3.4 ± 1.2	0.007 ± 0.001
CRA	200,000	13.0 ± 1.9	0.312 ± 0.07
	700,000	14.8 ± 3.0	0.049 ± 0.01

<sup>a</sup> Mean ± SE.

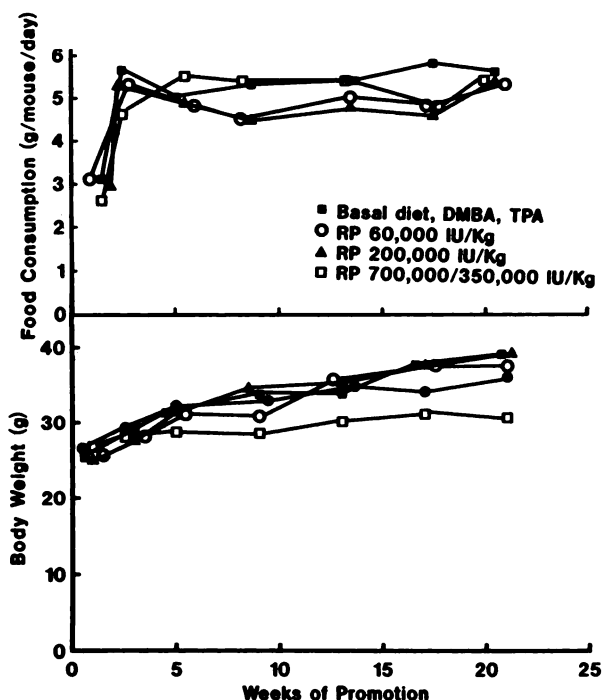


Fig. 3. The effect of retinyl palmitate supplementation on body weight and food consumption. Mice were treated as described in Table 1.

37%, or 65% inhibition of papillomas/mouse, respectively, at 21 wk of promotion. Taking into account the biopotency of retinyl palmitate, 1.8 IU/ $\mu$ g (13), and the mean daily intake of food, the average daily consumption of retinyl palmitate was approximately 155  $\mu$ g, 513  $\mu$ g, or 1556  $\mu$ g for 35 days and 1024  $\mu$ g for the next 113 days, in the 3 RP groups, respectively.

Bollag (14) demonstrated that retinoic acid at a dose of 0.2 mg/g mouse given by stomach tube at intervals of 14 days during promotion by croton oil resulted in a 40% inhibition of papillomas at 20 wk of promotion. Using topical applications of 5.1 or 0.51 mg of retinoic acid per mouse twice weekly during promotion by TPA, Verma obtained a 78 or 61% inhibition of skin papillomas, respectively, at 19 wk of promotion (3). Due to the differences in experimental treatment, a quantitative comparison of chemopreventive efficacy of retinyl palmitate with retinoic acid is not possible, although they gave roughly similar results.

Caloric restriction has been shown to inhibit tumorigenesis (15). Boutwell (12) found that diet restriction inhibits mouse croton oil-induced skin tumor promotion, but does not inhibit initiation by DMBA. A 40% reduction in calories yielded a 70% inhibition of papillomas per mouse at 16 wk. Ip (16) has observed that dietary restriction resulting in a 10% decrease in body weight over a 24-wk period inhibited DMBA-induced

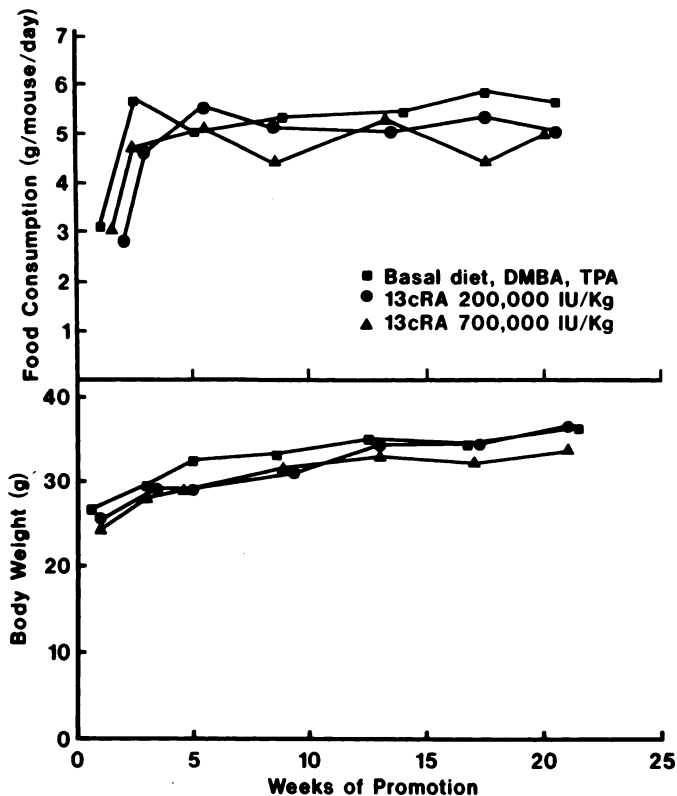


Fig. 4. The effect of supplementation with 13-cis-retinoic acid on body weight and food consumption. Mice were treated as described in Table 1.

mammary tumorigenesis in Sprague-Dawley rats by 20%. In our experiment, it is possible that the inhibition of tumor promotion observed in the mice fed 350,000 IU of retinyl palmitate per kg of diet could be partly due to the 16% decrease in body weight. However, body weight was normal at the 200,000-IU/kg dose level of retinyl palmitate, and this dose level still yielded a marked reduction of tumor burden.

It is noteworthy that the slope of the line for papillomas/mouse became negative after 16 wk of promotion in the mice treated with the highest dose of RP. This decrease in papillomas at 16 wk was due to visible and palpable regression of tumors, not to deaths of animals bearing multiple papillomas. These results are consistent with a similar regression of papillomas observed by Bollag after 13 wk of retinoic acid treatment during croton oil-induced tumor promotion (14).

When 13-cis-retinoic acid was applied topically, it was almost as effective as retinoic acid in preventing the appearance of mouse skin papillomas promoted by TPA (3). However, dietary CRA did not prevent tumors from appearing, although it did reduce the weight of tumor burden per mouse. These results agree with those obtained by Verma (17) who also used CD-1 mice. Epstein reported preliminary studies in which 13-cis-retinoic acid p.o. did not reduce UV-induced skin cancer formation (18). There was no significant reduction in food consumption or body weight of mice fed diets supplemented with CRA, so that caloric restriction did not account for this reduction of tumor burden per mouse.

The levels and distribution of retinol and retinyl palmitate in selected tissues of the mice fed diets supplemented with RP were measured by high-performance liquid chromatography at 25 wk of promotion. The data have been published in a separate paper (19) and show that there was a dose-dependent increase in the accumulation of both RP and retinol in the tumor tissue and skin. Localized retinoids may induce differentiation within

papillomas, as they do with epidermal papilloma cells *in vitro* (20).

The possibility of increased prophylaxis during the late stages of carcinogenesis is of obvious clinical significance due to the growing recognition of preneoplastic lesions in many types of cancers. The quest for chemopreventive and therapeutic agents which are active at doses showing minimal toxicity warrants further investigation.

#### ACKNOWLEDGMENTS

We thank Lisa Werner, Lucia Tobin, and Yumiko Moriguchi for dietary preparation and animal care; Norma Seaver and Janet Gruhn for technical assistance; and Sally Anderson for expert secretarial assistance. We appreciate the assistance of Dr. E. M. Peng and Dr. D. Alberts in measuring the dietary retinyl palmitate and 13-*cis*-retinoic acid.

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