

Influence of the Duration of Topical 13-*cis*-Retinoic Acid Treatment on Inhibition of Mouse Skin Tumor Promotion¹

Helen L. Gensler,² Dalice A. Sim, and G. Tim Bowden

Department of Radiation Oncology and Cancer Center, University of Arizona Health Sciences Center, Tucson, Arizona 85724

ABSTRACT

The effect of the time and duration of retinoid treatment on the inhibition of Stage II tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was studied in CD-1 mice. All mice were initiated with 400 nmol of benzo(a)pyrene and received Stage I tumor promotion (3.2 nmol of TPA twice weekly for 2 wk). Animals were then randomized into groups which received 13-*cis*-retinoic acid during early, middle, or late Stage II promotion. 13-*cis*-Retinoic acid pretreatments starting on Day 1, Wk 8, or Wk 23 of Stage II promotion resulted in 47, 28, or 19% inhibition, respectively, of TPA-induced tumor formation. One-half of the mice receiving 13-*cis*-retinoic acid at Day 1 or Wk 8 were removed from the retinoid treatments at Wk 23, the time of cessation of TPA promotion. The inhibition of tumor formation remained constant during the 15-wk observation period after cessation of retinoid treatment, suggesting that retinoid inhibition of mouse skin tumor promotion is stable in the absence of further promotion and preceded the step of irreversible conversion of promoter dependence to promoter independence.

INTRODUCTION

The capacity of retinoids to prevent mouse skin tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate and prevent epithelial cancers in experimental animals has been well documented (1-6). However, the mechanism(s) for this activity has not yet been defined. There is *in vitro* evidence that tumor promoters induce DNA damage (7, 8) and that effects of retinoids could lead to a decrease in promoter-induced DNA damage. For example, retinoids have been found to prevent the TPA³-induced release of active oxygen species by leukocytes (9, 10) which can lead to DNA single-strand scission in adjacent cells (7, 8). Damage to DNA alone has been shown to initiate the neoplastic transformation process (11). DNase I, when delivered into Syrian hamster embryo cells by means of liposomes, transformed the cells to become tumorigenic when injected into newborn Syrian hamsters. If a major mechanism for retinoid prevention of mouse skin tumor promotion *in vivo* is by reduction of DNA damage, then the inhibition would be stable in the absence of further promotion. The retinoid CRA has been shown to inhibit mouse skin tumor promotion induced by TPA. However, the question of the stability of this inhibitory effect has not been answered. The goal of the research presented in this paper was to determine the stability of the tumor inhibitory effect of CRA in the absence of further promotion. In addition to considering reversibility of CRA-induced inhibition of tumor promotion, we wished to compare the levels of inhibition achieved when CRA was applied at: (a) the beginning of Stage II (1) of tumor promotion (Day 1); or (b) the time of the initial appearance of tumors (Wk 8); or (c) the time of initial conversion of papillomas to carcinomas (Wk 23). The question of the stability of the chemopreventative effects of the

retinoids in the presence and absence of subsequent promotion is of great importance in considering the eventual use of these agents in preventing human cancer.

MATERIALS AND METHODS

Materials. Female CD-1 mice were purchased from the Charles River Breeding Laboratory (Wilmington, MA) and were used for experimentation at 8 wk of age. Benzo(a)pyrene was purchased from Aldrich Chemical Co. (Milwaukee, WI). TPA was obtained from Chemical Carcinogenesis Co. (Eden Prairie, MN). 13-*cis*-Retinoic acid was supplied by Hoffman-LaRoche (Nutley, NJ).

Treatment of Mice. Animals were housed in stainless steel cages (5 mice/cage) in a light- and temperature-controlled room. Food and water were available *ad libitum*. Mice were shaved 2 days before initiation, and only those mice in the resting phase of the hair cycle were used for experimentation. The solutions of B(a)P, TPA, and CRA were prepared in spectrograde acetone and applied to individual mice in a volume of 0.2 ml. The same volume of acetone was applied to the control mice 30 min before each TPA treatment. All solutions were prepared just before treatment of the animals. The 13-*cis*-retinoic acid was stored in powder form, protected from light, at -80°C. It was tested periodically for chemical purity by high-pressure liquid chromatographic analysis in Dr. Alberts' laboratory at this university. The acetone solutions were prepared in subdued light just before treatments and were applied under subdued lighting. The experimental design is shown in Fig. 1. All mice were initiated with a topical application of 400 nmol of B(a)P. One wk later Stage I tumor promotion (1) was begun and consisted of 3.2 nmol of TPA applied topically twice weekly for 2 wk. Then, animals were randomized into 7 groups of 20 mice each, and Stage II tumor promotion was started. One group of mice was treated with solvent alone, and the remaining animals were treated with TPA (8 nmol applied twice weekly for 23 wk). At this time, 2 groups of the TPA-treated mice received a pretreatment of CRA (17 nmol applied 30 min before each TPA treatment). During the eighth wk of Stage II tumor promotion, 2 groups of mice receiving TPA began to receive the CRA pretreatments. At the 23rd week of Stage II tumor promotion, all TPA treatments were stopped. This time was selected because previous experiments had shown that the papillomas were tumor promoter independent at this time (Footnote 4; confirmed in Fig. 2). On Wk 23, another group of mice began twice weekly treatments with CRA, and retinoid treatments were stopped on one-half of the mice which had previously been receiving CRA.

Statistical Analysis of Tumor Data. The significance of the difference in the tumor data obtained from the control and 13-*cis*-retinoic-treated mice was determined with a one-sided Wilcoxon rank sum test. The Wilcoxon test was used because the number of tumors per mouse in the TPA-treated control mice was not normally distributed. The significance of differences in mean tumor weight per mouse between treated and control mice was calculated using the Student *t* test.

RESULTS

Stage II tumor promotion by twice weekly applications of 8 nmol of TPA was inhibited by pretreatment with 17 nmol of CRA, as shown in Fig. 2 and Table 1. When the retinoid pretreatments started on the first day or eighth wk of Stage II tumor promotion, there was a 47 or 28% inhibition, respectively, of tumor formation at wk 27 to 38. When the retinoid

Received 9/16/85; revised 1/23/86; accepted 3/12/86.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This investigation was supported by NIH Grant CA-27502.

² To whom requests for reprints should be addressed.

³ The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; CRA, 13-*cis*-retinoic acid; B(a)P, benzo(a)pyrene; DMBA, 7,12-dimethylbenz(a)-anthracene.

⁴ Unpublished results.

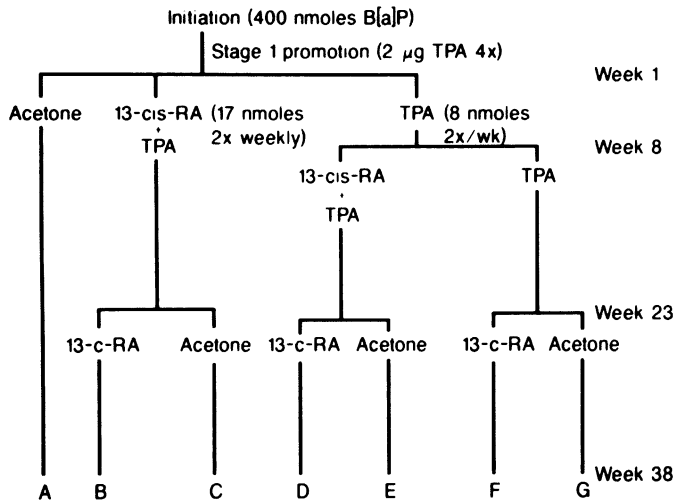


Fig. 1. Experimental design. All mice were initiated with a topical application of B(a)P and received 4 applications of 2 µg of TPA (Stage I tumor promotion). Treatments varied as shown during Stage II tumor promotion. The experiment was terminated at Wk 38. 13-cis-RA and 13-c-RA, CRA.

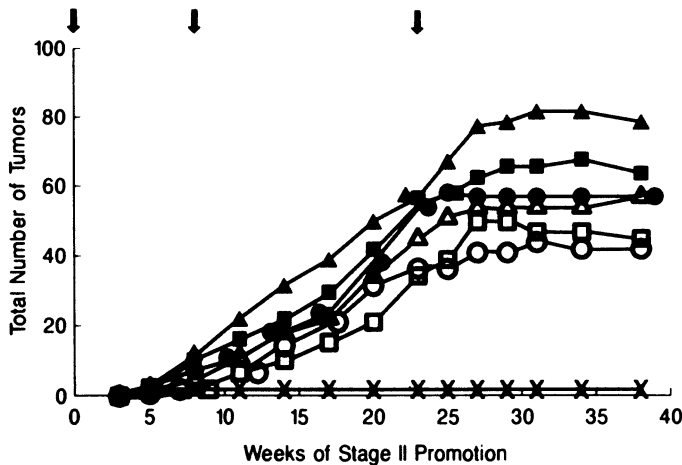


Fig. 2. The effect of time and duration of exposure to 13-cis-retinoic acid on mouse skin tumor formation. Mice were initiated and promoted as described in "Materials and Methods." Groups of 20 animals each received topical applications of: acetone from Wk 1 to Wk 38 (x); TPA promotion plus CRA from Wk 1 to 38 (O); TPA promotion plus CRA from Wk 1 to 23 (□); TPA promotion plus CRA from Wk 8 to 38 (●); TPA promotion plus CRA from Wk 8 to 23 (Δ); TPA promotion plus CRA from Wk 23 through 38 (■); or Stage II tumor promotion without CRA treatment (▲). The arrows indicate times when 13-cis-retinoic acid treatments began in different groups of mice.

treatments were delayed until the 23rd week of Stage II tumor promotion, there was a 19% inhibition of tumor formation. Thus, tumor inhibition, at the plateau level of tumors per group of mice, increased with increasing length of retinoid treatment.

Since the number of tumors per mouse was not normally distributed, we used a one-sided Wilcoxon rank sum test to determine whether mice undergoing the earliest retinoid treatment had a lower median number of tumors than those in the control group. Based on the numbers of tumors presented in Table 1 and Fig. 2, we found a significantly lower median ($P < 0.05$) at the plateau level. The inhibition of tumor formation remained relatively constant during the 15-wk observation period after cessation of retinoid treatment, as shown in Fig. 2 and Table 1. It is noteworthy that this observation period is 2 to 3 times as long as the time-to-tumor appearance in early Stage II tumor promotion. The rate of tumor formation as well as the final number of tumors was reduced by 13-cis-retinoic acid, although the percentage of mice bearing tumors was similar in retinoid- and acetone-treated mice (Fig. 3).

Not only the number of tumors, but also the mean weight of tumors per mouse showed a time-of-treatment response to 13-cis-retinoic acid treatment, as shown in Fig. 4. The mean weights of tumors per mouse in the groups treated with CRA beginning on Wk 1 or 8 of Stage II promotion were significantly different at the 95% confidence level from control values (by the Student *t* test). No significant difference was found in mean tumor weight per mouse between control mice and those treated with CRA starting on Wk 23 of Stage II promotion.

DISCUSSION

The inhibition of TPA-promoted mouse skin tumors was greatest with the earliest application of 13-cis-retinoic acid (Fig. 2). Although the extent of tumor prevention varied with the time of onset of retinoid treatment, there was inhibition even when CRA was first applied as late as the time of conversion of some of the papillomas to carcinomas. It appears, therefore, that there is not a specific time during late promotion which is retinoid sensitive. It is noteworthy, in this regard, that the slope of the curve of the accumulation of tumors was still positive at the time when the last group began to receive CRA. Therefore, some tumors were still in an early step in the tumorigenic pathway. The present experiment cannot distinguish if there are several steps during promotion which are retinoid sensitive, or if there is one retinoid step through which each potential tumor cell must pass.

The inhibition of tumor promotion was stable during 15 wk after the cessation of CRA and TPA treatments, suggesting that CRA prevented the irreversible molecular mechanism underlying TPA-induced promotion and demonstrating that antiproliferative action of 13-cis-retinoic acid was not its major pathway of prevention of TPA-promoted mouse skin tumors. These *in vivo* results are in contrast to *in vitro* findings with the

Table 1 Effects of time and duration of CRA treatment on Stage II promotion induced by TPA

Initiation was with 400 nmol of B(a)P and was followed 1 wk later by twice weekly applications of 2 µg of TPA for 2 wk (Stage I). Starting on the next week, the mice received twice weekly applications of 8 nmol of TPA for 23 wk (Stage II). CRA was given during selected weeks throughout or immediately after Stage II promotion, as indicated in Table 1 and Fig. 1. There were 20 mice in each group.

Observation time, wk of Stage II promotion	No. of tumors/group						
	Acetone	TPA	13-cis-Retinoic acid treatment (wk)				
			1-38	1-23	8-38	8-23	23-38
23	2 (0.1 ± 0.3) ^a	57 (2.85 ± 2.4)	36 (1.8 ± 2.0)	34 (1.7 ± 2.5)	54 (2.7 ± 3.5)	45 (2.3 ± 2.8)	55 (2.8 ± 3.0)
25	2 (0.1 ± 0.3)	67 (3.35 ± 3.3)	36 (1.8 ± 2.0)	39 (1.95 ± 2.6)	58 (2.9 ± 3.6)	51 (2.55 ± 2.8)	58 (2.9 ± 3.1)
27	2 (0.1 ± 0.3)	78 (3.9 ± 3.7)	41 (2.05 ± 2.7)	50 (2.5 ± 3.7)	57 (2.85 ± 3.6)	54 (2.7 ± 2.8)	63 (3.15 ± 2.7)
29	2 (0.1 ± 0.3)	79 (3.95 ± 3.8)	41 (2.05 ± 2.7)	50 (2.5 ± 3.7)	57 (2.85 ± 3.6)	54 (2.7 ± 2.8)	66 (3.3 ± 2.8)
31	2 (0.1 ± 0.3)	82 (4.1 ± 4.3)	44 (2.2 ± 2.9)	47 (2.35 ± 3.4)	57 (2.85 ± 3.6)	54 (2.7 ± 2.8)	66 (3.3 ± 2.8)
34	2 (0.1 ± 0.3)	82 (4.1 ± 4.3)	42 (2.1 ± 2.4)	47 (2.35 ± 3.4)	57 (2.85 ± 3.6)	54 (2.7 ± 2.8)	68 (3.4 ± 3.2)
38	2 (0.1 ± 0.3)	79 (3.95 ± 4.2)	42 (2.1 ± 2.4)	45 (2.25 ± 2.9)	57 (2.85 ± 3.6)	57 (2.85 ± 2.9)	64 (3.2 ± 2.6)

^a Numbers in parentheses, mean ± SD.

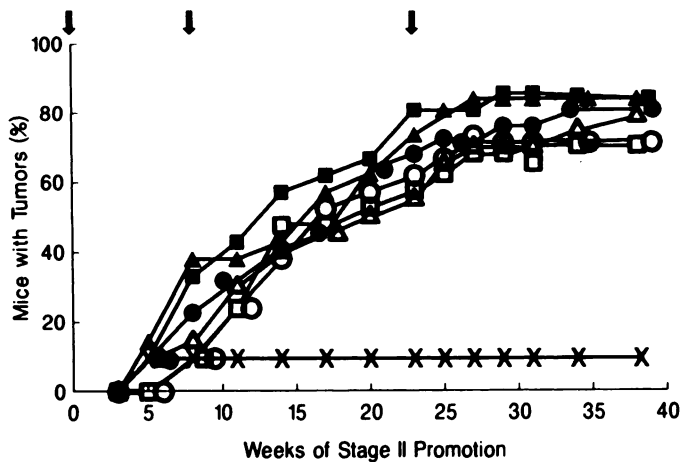


Fig. 3. The effect of time and duration of exposure to 13-cis-retinoic acid on the incidence of mouse skin tumors promoted with TPA. The symbols are identical with those used in Fig. 2.

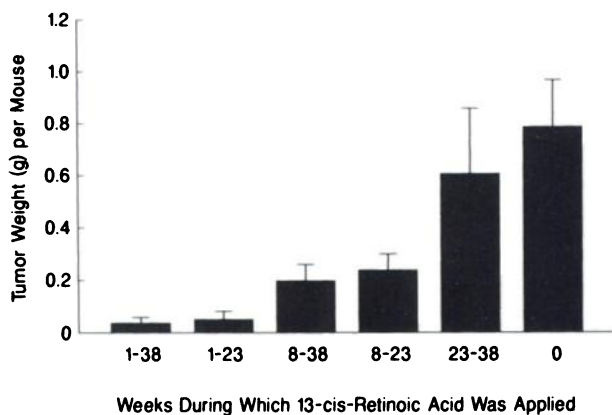


Fig. 4. The influence of time and duration of exposure to 13-cis-retinoic acid on weight of tumor burden per mouse. Columns, mean; bars, SE.

mouse epidermal cell line JB6 (12), in which an antiproliferative effect of β -all-trans-retinoic acid was able to account for the observed reduction in TPA-induced colony formation in soft agar. In agreement with these *in vivo* murine results, topical retinoid acid therapy (0.05% solution applied daily for 12 wk) on human multiple dysplastic nevi, which are considered to be preneoplastic lesions, resulted in loss of dysplasia with no recurrence at 7 or 8 mo after therapy.⁵

Verma (13) has found that retinoic acid does not extend protection to TPA treatments applied to mice after cessation of retinoid treatment. In Verma's study, retinoic acid (17 nmol) was applied before each TPA treatment for the first 10 wk of promotion, after which TPA treatments alone were continued for a total promotion duration of 30 wk. The rate of tumor appearance after cessation of retinoic acid treatment was almost equal to the rate in TPA control mice. If retinoid inhibition of tumors were due to a reversible block at a step preceding the appearance of a promoter-dependent tumor, then an increased rate of tumor appearance should follow removal of the retinoid in the continued presence of promotion. Thus, there would be the positive control rate of promotion plus the addition of the now unblocked clones in the tumorigenic pathway. Verma found no such increase in the rate of tumor appearance after removal of retinoid treatment in the continued presence of promotion. Taken together these results are consistent with the

interpretation that an effective retinoid can completely prevent a step(s) which precedes the irreversible conversion of a promoter-dependent tumor to a promoter-independent tumor, but it does so only as long as the retinoid is present.

It is possible that a stable prevention of tumor formation by retinoids can occur only when retinoid treatment is concomitant with a susceptible step(s) in promotion. It is noteworthy that mouse skin tumor promotion by some agents, such as anthralin (14) or benzoyl peroxide (15), bypasses the retinoid-sensitive step(s). In addition, complete carcinogenesis is not as effectively inhibited by retinoids as is TPA-induced promotion, as has been shown in the mouse skin tumor system (2). Retinoic acid applied in conjunction with TPA following initiation with DMBA inhibited the formation of skin papillomas. However, retinoic acid applied in conjunction with each weekly application of 0.2 μ mol DMBA did not inhibit DMBA-induced skin tumors. In rat mammary carcinoma induction by 1-methyl-1-nitrosourea, retinyl acetate acts as an effective chemopreventive agent, but withdrawal of the retinoid (in the absence of further 1-methyl-1-nitrosourea treatment) results in a rapid increase in the appearance of mammary cancers (16). Preliminary results using retinoids for chemotherapy in human patients with multiple basal cell carcinomas, actinic keratoses, and keratoacanthomas suggest that postregression maintenance therapy is mandatory to prevent appearance of new tumors (17). Thus, it appears likely that only promoter-dependent lesions will prove to be effectively inhibited from tumor progression by retinoids, and that this inhibition will not extend to subsequent promotion.

A number of the mechanisms proposed for retinoid chemopreventive activity could yield an inhibition which is stable in the absence of subsequent promotion. These mechanisms include effects on differentiation (18-20), immunomodulation (21, 22), oncogene regulation (19), or prevention of an irreversible but as yet undefined step of promotion. In relation to the last possibility, release of active oxygen species by TPA-stimulated leukocytes induced DNA single-strand scission in adjacent target cells (7, 8). Cells exposed to activated leukocytes can be tumorigenic in nude mice (23). Since retinoic acid can inhibit the release of active oxygen species from leukocytes (9, 10), it is possible that retinoids prevent promotion by blocking leukocyte-induced DNA strand scission. Breaks in DNA, induced by DNase I in Syrian hamster embryo cells, have been shown to lead to mutations, chromosomal aberrations, hyperdiploidy, and tumorigenicity (11). Three factors provide evidence that DNA strand scission can be important in the carcinogenic pathway: (a) the acquisition of neoplastic transformation in cells after they were exposed to an agent that induces DNA chain scission (11); (b) the striking susceptibility to cancer in diseases which involve genetic instability (24); and (c) the high level of chromosomal aberrations in fresh tumor cells (25).

In summary, a sustained inhibition of papilloma formation was observed throughout a 15-wk period after withdrawal of 13-cis-retinoic acid and TPA treatment, suggesting that this chemoprevention was stable in the absence of further promotion and preceded the step of irreversible conversion of promoter dependence to promoter independence.

ACKNOWLEDGMENTS

We thank Dr. Ajit Verma, Dr. Norman Levine, Dr. Libby Edwards, and Dr. Frank Meyskens for sharing with us their results before publication, and Sally Anderson for expert secretarial assistance.

⁵ N. Levine, L. Edwards, and F. L. Meyskens. Topical retinoic acid therapy for multiple dysplastic nevi, submitted for publication.

REFERENCES

1. Slaga, T. J., Fischer, S. M., Nelson, K., and Gleason, G. L. Studies on the mechanism of skin tumor promotion: evidence for several stages in promotion. *Proc. Natl. Acad. Sci. USA*, **77**: 3659-3663, 1980.
2. Verma, A., Conrad, E. A., and Boutwell, R. K. Differential effects of retinoic acid and 7,8-benzoflavone on the induction of mouse skin tumors by the complete carcinogenesis process and by the initiation-promotion regimen. *Cancer Res.*, **42**: 3519-3525, 1982.
3. Moon, R. C., Grubbs, C. J., Sporn, M. B., and Goodman, D. G. Retinyl acetate inhibits mammary carcinogenesis induced by *N*-methyl-*N*-nitrosourea. *Nature (Lond.)*, **267**: 620-621, 1977.
4. Sporn, M. B., Squire, R. A., Brown, C. C., Smith, J. M., Wenk, M. L., and Springer, S. 13-*cis*-Retinoic acid: inhibition of bladder carcinogenesis in the rat. *Science (Wash. DC)*, **195**: 487-489, 1977.
5. Grubbs, C. J., Moon, R. C., Squire, R. A., Farrow, G. M., Stinson, S. F., Goodman, D. G., Brown, C. C., and Sporn, M. B. 13-*cis*-Retinoic acid: inhibition of bladder carcinogenesis induced in rats by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. *Science (Wash. DC)*, **198**: 743-744, 1977.
6. Smith, D. M., Rogers, A. E., and Newberne, P. M. Vitamin A and benzo(a)pyrene carcinogenesis in the respiratory tract of hamsters fed a semisynthetic diet. *Cancer Res.*, **35**: 1485-1488, 1975.
7. Birnboim, H. C. Importance of DNA strand-break in tumor promotion. *In: O. F. Nygaard and M. E. Simic (eds.), Radioprotectors and Anticarcinogens*, pp. 539-556. New York: Academic Press, 1983.
8. Dutton, D. R., and Bowden, G. T. Indirect induction of a clastogenic effect in epidermal cells by a tumor promoter. *Carcinogenesis (Lond.)*, **6**: 1279-1284, 1985.
9. Witz, G., Goldstein, B. D., Amoroso, M., Stone, D. S., and Troll, W. Retinoid inhibition of superoxide anion radical production by human polymorphonuclear leukocytes stimulated with tumor promoters. *Biochem. Biophys. Res. Commun.*, **97**: 883-888, 1980.
10. Kensler, T. W., and Trush, M. A. Inhibition of phorbol ester-stimulated chemiluminescence in human polymorphonuclear leukocytes by retinoic acid and 5,6-epoxyretinoic acid. *Cancer Res.*, **41**: 216-222, 1980.
11. Zajac-Kaye, M., and Ts'o, P. O. DNase I encapsulated in liposomes can induce neoplastic transformation of Syrian hamster embryo cells in culture. *Cell*, **39**: 427-437, 1984.
12. Gensler, H. L., Matrisian, L. M., and Bowden, G. T. Effect of retinoic acid on the late-stage promotion of transformation in JB6 mouse epidermal cells in culture. *Cancer Res.*, **45**: 1922-1925, 1985.
13. Verma, A. Inhibition of mouse skin tumor promotion by retinoids. *In: K. N. Prasad (ed.), Advances in Vitamins, Nutrition, and Cancer*. Basel: Karger, in press, 1986.
14. Gensler, H. L., and Bowden, G. T. Influence of 13-*cis*-retinoic acid on mouse skin tumor initiation and promotion. *Cancer Lett.*, **22**: 71-75, 1984.
15. Slaga, T. J., and Fischer, S. M. Strain differences and solvent effects in mouse skin carcinogenesis experiments using carcinogens, tumor initiators, and promoters. *Prog. Exp. Tumor Res.*, **26**: 85-109, 1983.
16. Thompson, H. J., Becci, P. J., Brown, C. C., and Moon, R. C. Effect of the duration of retinyl acetate feeding on inhibition of 1-methyl-1-nitrosourea-induced mammary carcinogenesis in the rat. *Cancer Res.*, **39**: 3977-3980, 1979.
17. Peck, G. L. Synthetic retinoids in dermatology. *In: M. B. Sporn, A. B. Roberts, and D. S. Goodman (eds.), The Retinoids*, Vol. 2, pp. 392-411. New York: Academic Press, 1984.
18. Jetten, A. M. Modulation of cell growth and differentiation by retinoids and their mechanisms of action. *In: F. L. Meyskens and K. N. Prasad (eds.), Modulation and Mediation of Cancer by Vitamins*, pp. 177-186. Basel: Karger, 1983.
19. Westin, E. H., Gallo, R. C., Arya, S. K., Eva, A., Souza, L. M., Baluda, M. A., Aaronson, S. A., and Wong-Staal, F. Differential expression of the *amv* gene in human hematopoietic cells. *Proc. Natl. Acad. Sci. USA*, **79**: 2194-2198, 1982.
20. Roberts, A. B., and Sporn, M. B. Cellular biology and biochemistry of the retinoids. *In: M. B. Sporn, A. B. Roberts, and D. S. Goodman (eds.), The Retinoids*, Vol. 2, pp. 209-286. New York: Academic Press, 1984.
21. Lotan, R., and Dennert, G. Stimulatory effects of vitamin A analogs on induction of cell-mediated cytotoxicity *in vivo*. *Cancer Res.*, **39**: 55-58, 1979.
22. Moriguchi, S., Werner, L., and Watson, R. High dietary vitamin A and cellular immune functions in mice. *Immunology*, **56**: 169-177, 1985.
23. Weitzman, S. A., Weitberg, A. B., Clark, E. P., and Stossel, T. P. Phagocytes as carcinogens: malignant transformation produced by human neutrophils. *Science (Wash. DC)*, **227**: 1231-1233, 1985.
24. German, J. Patterns of neoplasia associated with the chromosome-breakage syndromes. *In: J. German (ed.), Chromosome Mutation and Neoplasia*, pp. 97-134. New York: Alan R. Liss, Inc., 1983.
25. Mitelman, F. *Catalog of Chromosome Aberrations in Cancer*. New York: Alan R. Liss, Inc., 1985.