

Stimulatory Effects of Vitamin A Analogs on Induction of Cell-mediated Cytotoxicity *in Vivo*¹

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ABSTRACT

Previously, we observed that pretreatment of mice with low doses (25 to 300 $\mu\text{g}/\text{mouse}/\text{day}$) of the antineoplastic agent, β -all-*trans*-retinoic acid (β -RA), for 5 days before challenge with allogeneic tumor cells resulted in stimulated induction of cell-mediated cytotoxicity (CMC) but that higher doses (≥ 500 $\mu\text{g}/\text{mouse}/\text{day}$) suppressed CMC. The present study examined the ability of three other less toxic retinoids to stimulate CMC. Administration of 25-, 100-, 300-, or 800- $\mu\text{g}/\text{mouse}/\text{day}$ i.p. doses of β -RA, trimethylmethoxyphenyl analog of β -RA, 13-*cis*-retinoic acid, or retinyl palmitate daily for 5 days into C57BL/6 mice stimulated CMC to 8- to 10-fold after challenge with suboptimal immunogen inoculum (10^6 S194 myeloma cells/mouse). When retinoid-treated mice were challenged with a higher number of tumor cells (3×10^6 or $10^7/\text{mouse}$), CMC was also enhanced; however, it was to a low degree (2- to 4-fold). Optimal stimulation of CMC by β -RA occurred at 100 $\mu\text{g}/\text{mouse}/\text{day}$, while at 800 $\mu\text{g}/\text{mouse}/\text{day}$ CMC was somewhat inhibited. In contrast, the three other retinoids did not suppress but rather stimulated CMC even better at the highest dose tested. The trimethylmethoxyphenyl analog exhibited a higher stimulatory effect on CMC than did the other retinoids, especially in mice challenged with optimal immunogen doses. These results demonstrate that, in addition to β -RA, other retinoids are capable of enhancing CMC. This property may in part mediate their reported antineoplastic activity.

INTRODUCTION

Vitamin A analogs [retinoids (26, 27)] exhibit antineoplastic activity against epithelial tumors induced by chemical carcinogens *in vivo* (1, 2, 14, 21, 22, 24, 26-28) or *in vitro* (5, 7, 17). Inhibition of the growth and development of transplantable tumors both *in vivo* (12, 23, 30) and *in vitro* (18) was also demonstrated. Several studies proposed that, since certain retinoids can stimulate immune responses (6, 11, 16, 20, 25), some of their antitumor effects could be mediated indirectly via enhancement of host antitumor immune response (12, 13, 16, 19, 23, 29). Indeed, we have recently demonstrated that β -RA³ administered i.p. to

C57BL/6 mice at low doses (≤ 300 $\mu\text{g}/\text{mouse}/\text{day}$) for 5 days stimulated the induction of CMC against allogeneic BALB/c-S194 tumor cells (10). The enhanced CMC was specific for the alloantigen (H-2^d) expressed on the S194 tumor cells used for immunization, and the cytotoxicity was mediated by T-cells, since it could be abrogated by treating the spleen cells with anti- θ and complement (8). At higher doses (≥ 500 $\mu\text{g}/\text{mouse}/\text{day}$), β -RA suppressed CMC and caused systemic toxicity (10). Others have encountered similar toxic effects (2, 15, 30); therefore, retinoids with lower toxicity or with better therapeutic ratio [quotient between weekly dose required for 50% inhibition of tumor growth and minimal daily dose causing toxic symptoms (2)] were sought (26, 27). Indeed, synthetic analogs of retinoic acid, 13-RA and TMMP, were found to be less toxic (15, 21, 22, 28) or to have a better therapeutic ratio (2) than did β -RA. We therefore tested the ability of these synthetic retinoids to stimulate CMC and compared their effect with that of β -RA and RP.

MATERIALS AND METHODS

Animals. C57BL/6 mice were obtained from the NCI-Frederick Cancer Research Center, Frederick, Md. They were used for the present experiments at the age of 7 weeks; average body weight was 21 ± 1.5 (S.D.) g.

Cells. The BALB/c myeloma S194 was grown in Dulbecco's modified Eagle's minimal essential medium containing 10% heat-inactivated horse serum.

Retinoids. β -RA, 13-RA, RP, and TMMP were kindly provided by Hoffmann-La Roche Inc. (Nutley, N. J.). Retinoids had been suspended in corn oil immediately before they were injected i.p. (0.1 ml/mouse) according to the schedule and dosage indicated in the next section.

Induction of CMC. CMC mediated by thymus-derived (T) lymphocytes (8) was induced by i.p. injection of BALB/c-S194 myeloma cells into C57BL/6 mice as described previously (10). In the particular experiment presented here, 153 mice were divided randomly into 4 groups of 36 mice each, to be treated with each of the 4 retinoids, and 1 group of 9 mice to serve as control. The latter group received i.p. corn oil injections, whereas each of the 4 other groups was further divided into 4 subgroups, and each of the 9 mice in each subgroup received daily i.p. injections of one of the 4 retinoids, either 25, 100, 300, or 800 $\mu\text{g}/\text{mouse}/\text{day}$, for 5 days. On Day 7, each of the subgroups, as well as the 9 control mice, were divided into 3 subgroups, each consisting of 3 mice, before i.p. injections of 10^6 , 3×10^6 , or 10^7 S194 cells/mouse to each of these subgroups, respectively. At the peak of the CMC response in this system (7 days after immunogen injection), the mice were sacrificed, and their

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³ The abbreviations used are: β -RA, β -all-*trans*-retinoic acid; CMC, cell-mediated cytotoxicity; 13-RA, 13-*cis*-retinoic acid; TMMP, trimethylmethoxyphenyl analog of β -all-*trans*-retinoic acid; RP, retinyl palmitate; a/t, attacker/target cell ratio.

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spleens were removed, minced washed, and assayed for CMC.

Assay of CMC. Spleen cells from control or retinoid-treated mice (attacker cells) were assayed for cytotoxic activity on ⁵¹Cr-labeled target cells (S194) at a/t ratios of 10/1, 30/1, or 100/1 in Roswell Park Memorial Institute Medium 1640 containing 10% fetal calf serum as described previously (9). Cytotoxicity was expressed as percentage of specific ⁵¹Cr release according to the equation:

% cytotoxicity =

$$\frac{\text{Experimental } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}}{\text{Maximal } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}} \times 100$$

Maximal release was determined after freezing and thawing the target cells 3 times. The mean values of duplicate samples, as well as the S.E., were calculated. Since the CMC in control and in retinoid-treated mice was assayed on the same day using the same target cells, direct comparison between the cytotoxicities calculated for spleen cells from mice treated with the various retinoids and from untreated control mice could be made. The ratio of percentage of specific ⁵¹Cr released by spleen cells from retinoid-treated to that from control mice, at the same a/t ratio, was used to express quantitatively the stimulation of CMC by the retinoids.

RESULTS AND DISCUSSION

After preliminary experiments (not shown) had indicated that stimulation of CMC *in vitro*, previously demonstrated with β-RA (10), could be reproduced with other retinoids, the effects of 3 retinoids on induction of CMC *in vivo* were investigated and compared with the effect of β-RA. The retinoids 13-RA, RP, and TMMP (see Chart 1 for chemical structure) stimulated CMC as effectively as or even better than β-RA (Chart 2). Maximal stimulation by all retinoids was 8- to 10-fold at the suboptimal dose (10⁶/mouse) of the immunizing tumor cells (Chart 2A; Table 1). However, when

higher immunogen doses (3 × 10⁶ or 10⁷ cells/mouse) were used, CMC stimulation was only 1.4- to 3.9-fold (Chart 2, B and C; Table 1). The increase in CMC as presented in Table 1 is only a lower estimate of stimulation since, if one compares percentage of cytotoxicity in treated and control spleens at different a/t ratios, higher values are derived. For example, when CMC was induced by 10⁶ S194 cells/mouse (Chart 2A), the percentage of cytotoxicity was 6.4% in control spleens at a/t 100/1, while at optimal doses of each of the retinoids it was 14.4, 15.2, 12.8, and 9.6% for β-RA, 13-RA, RP, and TMMP, respectively, at a/t 10/1. This suggests that CMC in the retinoid-treated mice was actually stimulated by more than 10-fold. Similarly, the percentage cytotoxicity after induction with 3 × 10⁶ or 10⁷ S194 cells/mouse in control spleens at a/t 100/1 was lower than in retinoid-treated spleens at 30/1 indicating greater than 3-fold enhancement of CMC. Calculations of lytic units (4) would have been most appropriate for comparison between retinoid-treated and control spleens; however, since the highest percentage of cytotoxicity in control spleens was not greater than 25% at the highest immunogen dose, the construction of a significant percentage of cytotoxicity-*versus*-a/t ratio curve (4) was not possible.

The optimal dose of β-RA required for CMC enhancement

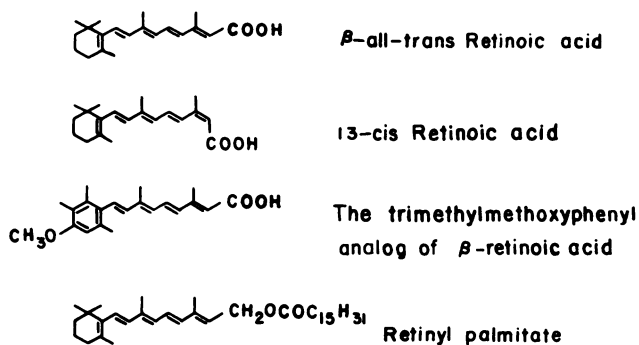


Chart 1. Chemical structure of the retinoids used in this study.

Chart 2. Stimulation of CMC in mice treated with various retinoids. Groups of 3 C57BL/6 mice were given daily i.p. injections of corn oil or various doses of the indicated retinoids for 5 days. On Day 7, mice were challenged i.p. with 10⁶ (A), 3 × 10⁶ (B), or 10⁷ (C) S194 tumor cells per mouse; after an additional 7 days, the mice were sacrificed, and their spleens were assayed for cytotoxic activity at attacker (spleen cells) to target (⁵¹Cr-labeled S194 myeloma cells) ratios of 10/1, 30/1, and 100/1 (each set of columns from left to right). The values represent percentage of cytotoxicity measured after 5 hr of incubation. Bars, S.E.

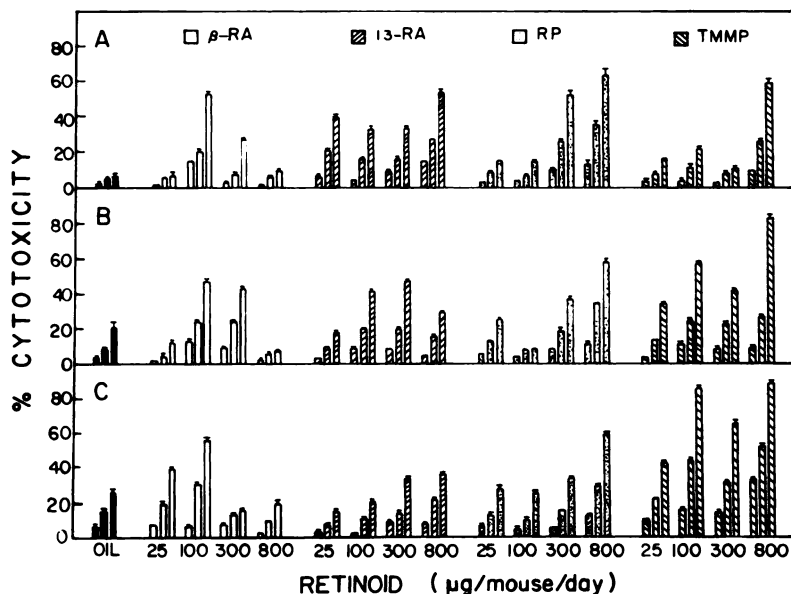


Table 1
Enhancement of CMC by various retinoids

The increase in percentage of ^{51}Cr released by spleen cells from retinoid-treated mice as compared with untreated controls at the optimal retinoid dose was calculated from the data in Chart 2.

Type	Retinoid		Increase in CMC (retinoid-treated/control) ^a
	Optimal dose ($\mu\text{g}/\text{mouse}/\text{day}$)	Immunogen dose (no. of S194 cells/mouse)	
β -RA	100	1×10^6	7.9
	100	3×10^6	2.2
	100	1×10^7	2.1
13-RA	800	1×10^6	8.3
	300	3×10^6	2.2
	800	1×10^7	1.4
RP	800	1×10^6	9.8
	800	3×10^6	2.7
	800	1×10^7	2.3
TMMP	800	1×10^6	9.0
	800	3×10^6	3.9
	800	1×10^7	3.4

^a Percentage of cytotoxicity measured with spleens from retinoid-treated/control at a/t 100/1.

was 100 $\mu\text{g}/\text{mouse}/\text{day}$. Higher doses, in particular 800 $\mu\text{g}/\text{mouse}/\text{day}$, caused some inhibition of CMC (Chart 2). In contrast, the other retinoids were usually more effective at the higher doses (800 $\mu\text{g}/\text{mouse}/\text{day}$). The TMMP analog of β -RA exhibited stronger stimulation of CMC at the higher immunogen doses than did the other retinoids tested (Table 1). Recent studies with β -RA (8) indicated that this retinoid acts at the induction step of the generation of T-cell-mediated cytotoxicity; however, the nature of the T-cell subpopulation that may be affected is not known. Although it is plausible to assume that the analogs of β -RA used in the present study may share a similar mechanism of action, this remains to be elucidated.

In addition to the effects on CMC, we also noticed that the mice that received β -RA, 800 $\mu\text{g}/\text{mouse}/\text{day}$, suffered from toxic symptoms such as loss of hair, scaling of the skin, and loss of weight. The 3 other retinoids administered at the same dose did not cause similar toxic effects. Thus, 13-RA, RP, and TMMP are less toxic to the mice and are capable of stimulating induction of CMC even better than is β -RA. It is plausible to assume that immunostimulation may account for part of their reported antineoplastic activity in addition to direct effect on tumor cells (3, 6, 12, 23, 29, 30). The lower toxicity of 13-RA, RP, and TMMP and their higher potency in CMC stimulation suggest that they may be more suitable for therapy, especially since they do not suppress CMC at high doses which may be required for direct effect on tumor cells. A recent report on studies with human patients with unresectable bronchogenic cancer demonstrated immune potentiating effects of both RP and 13-RA and concluded that the immune stimulatory properties of vitamin A are just as important as is the effect on the tumor itself (20). Although different immunological functions were stimulated in humans and experimental animals, the screening of new retinoids for derivatives with lower general toxicity and higher capacity to stimulate immune re-

sponses and inhibit tumor cell proliferation holds promise for clinical chemoimmunotherapy.

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