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Effect of an Aromatic Retinoic Acid Analog (Ro 10-9359) on Growth of Virus-Induced Papilloma (Shope) and Related Neoplasia of Rabbits

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Abstract—*An aromatic analog of retinoic acid (Ro* 10-9359), *a synthetic compound known to arrest development and growth of chemically-induced skin papillomas and carcinomas of mice, exerts a marked inhibitory effect on induction and development of virus-induced papilloma (Shope) of rabbit skin. The intramuscular administration of* 12.5, 50 *and* 200 mg/kg *given twice weekly during the induction phase of the neoplasia substantially inhibited the growth of the papilloma, this inhibition being dose-dependent. When the animals bearing well-established tumors were given a relatively large dose* (200 mg/kg) *of the compound, there was remarkable inhibition of the papillomatous growths and complete regression occurred in about* 60%. *The transplantable carcinomas Vx2 and Vx7, both of which originated from the Shope virus-induced papilloma, were less sensitive than their original papillomas to this treatment.*

INTRODUCTION

VITAMIN A and its natural and synthetic derivatives, the retinoids, play a significant role in cellular differentiation and to some extent in controlling growth of epithelial neoplasia. The mechanism of action has not been completely elucidated. In 1965, McMichael reported that the vitamin A palmitate retards the growth of Shope rabbit papilloma when this agent is administered systemically at a relatively large dose [1]. This finding led Bollag to search for synthetic molecules of retinoic acid analogs which would have a potent antitumor effect, yet without the toxicity known as the hypervitaminosis A syndrome. He used chemically-induced neoplasia of mice as the test system [2, 3].

Here we report the effect of one such potent synthetic retinoid, Ro 10-9359 [2], on the induction and growth of virus-induced papilloma (Shope) of rabbit skin [4] and also on the growth of transplantable carcinomas Vx2 [5] and Vx7 [6].

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MATERIALS AND METHODS

Animals

Random bred domestic rabbits (Japanese White) purchased from a local farm and weighing 2.0–2.5 kg were used in all experiments. Wild cottontail rabbits were trapped on an island in Mikawa Bay, Aichi Prefecture, Japan. These wild animals were primarily imported from the United States by courtesy of Drs. C. A. Evans and J. Thomsen of University of Washington, Seattle, between 1967 and 1968 and were let loose on the uninhabited island which was under the supervision of the Japan Monkey Center, Nagoya. The rabbits reproduced well in this environment and served as the source of Shope papilloma virus (SPV)-producing host animals.

Both wild and domestic rabbits were housed individually in metal cages and were maintained on a commercial pellet diet (Funahashi Farm, Inc.) and tap water *ad libitum*. The vitamin A content of the pellets was 11,500 i.u./kg.

Virus

The original source of the virus was the

Washington B strain of SPV, kindly provided by Dr. C. A. Evans. The viral agent was then propagated here in our laboratory in the cottontail rabbits. The papillomatous tissues induced by the SPV on the skin of cottontails were harvested, preserved in 50% buffered glycerin and stored at 4°C. The stock virus pool was prepared as 10% (w/v) tissue homogenate in phosphate buffered saline (PBS). It has a titer of 10^4 50% infectious dose (ID_{50}) per ml when titrated on domestic rabbit skin. The undiluted and 10× diluted extracts in PBS, designated as 10^{-1} and 10^{-2} SPV preparation, respectively, were used for the inoculation. The extracts were stored at −20°C until use.

Tumor induction

Inoculation with the SPV preparations was usually performed on the clipped and shaved skin on the dorsum of the domestic rabbits, using the scarification method [7]. The inoculation was given into two or four sites per animal, each with a dimension of 20 × 20 mm, employing 0.1 ml of SPV 10^{-1} (1000 ID_{50}) or SPV 10^{-2} (100 ID_{50}) per site. The tumors in the untreated controls were first evident 12–14 days post inoculation (p.i.). They were observed macroscopically and measurements were made every 2 days for 10 weeks after the tumor induction.

Most of the strains of transplantable Vx2 and Vx7 carcinomas were a gift from the late Dr. P. Rous. They arrived at our laboratory via the University of Washington (Dr. C. A. Evans) in 1966. Since then, they have been transplanted i.m. as 10 or 20% cell suspensions at intervals of 4–6 weeks. As evidence of their viral etiology, the two strains of neoplasia have been proven to harbor approximately 20 copies/diploid cells of SPV genome [8, 9]. In the present study a 20% (v/v) cell suspension containing 3×10^6 viable cells per ml was prepared in PBS and 1.0 ml of each strain of carcinomas was given i.d. at two sites on the dorsum of the domestic rabbits.

The approximate volume of the growths in both papillomas and transplantable carcinomas was estimated by the length × width × height. In some series of experiments, a more precise calculation of the tumor volume was attempted by preparing a replica of each tumor in dental plaster.

Chemical agents

Among the series of synthetic retinoids, ethyl all-trans-9-(4-methyoxy-2, 3, 6-trimethylphenyl) 3, 7-dimethyl-2, 4, 6, 8-monatetraenoate (Ro 10-9359) was selected because of its less toxic and increased antitumor property, as seen in the mouse experimental tumor system [10]. The agent was suspended in peanut oil at room temperature and was injected into the thigh muscle of rabbits twice weekly. In the assessment of its inhibitory effect on SPV tumorigenesis, doses of 12.5, 50 and 200 mg/kg of the compound in 2–5 ml of peanut oil were given. The control animals were given only the same amount of oily vehicle. The retinoid was a gift from Hoffmann-La Roche Co., Basel.

Assay of retinoids

The amount of retinoids in the various tissue specimens was determined by a sensitive and specific method of high-performance liquid chromatography [11] with some minor modifications. The assay was kindly performed by Dr. S. Maruyama of Sogo Biomedical Laboratories, Inc., Tokyo.

RESULTS

General observations

The effect of retinoid (Ro 10-9359) on the growth of domestic rabbits (Japanese White) when given i.m. twice weekly in a dose of 200 mg/kg resulted in suppression of increase in the body weight, as compared with the growth curves of the untreated controls. Such decline in growth was not evident until the 4th week (data not shown), after which the symptoms of so-called hypervitaminosis A were gradually manifested. These included loss of appetite and body weight, loss of hair usually beginning from the area around the nose, general weakness and occasional fracture of the bone in the vicinity of the injection site.

Effect on papilloma and growth

As shown in Table 1, the appearance of papillomas in retinoid-treated rabbits was significantly delayed as compared with the untreated controls. However, even with the highest dose employed (200 mg/kg), a complete inhibitory effect on the induction of the growth was not observed.

The effect of the retinoid (Ro 10-9359) on the growth of SPV-induced papillomas with respect to tumor volume is shown in Figs. 1a and 1b. In both groups of rabbits inoculated with SPV at a concentration of 10^{-1} and 10^{-2}, the suppressive action of the compound was usually dose-dependent.

Table 1. *Effect of retinoic acid analog* (*Ro* 10-9359) *on incubation period of virus-induced papilloma* (*Shope*)

Inoculum*	Treatment (mg/kg)	Average incubations (days)	(Incubation in days of individual tumors)
SPV 10^{-1}	0	10.50±0.55†	(11, 11, 10, 10, 11, 10)
	12.5	11.00±0.00	(11, 11, 11, 11, 11, 11)
	50	12.33±1.37	(14, 14, 12, 12, 11, 11)
	200	14.83±0.98	(16, 15, 15, 15, 15, 13)
SPV 10^{-2}	0	13.50±0.84	(15, 14, 13, 13, 13, 13)
	12.5	13.83±1.60	(17, 14, 13, 13, 13, 13)
	50	16.33±1.63	(19, 17, 17, 15, 15, 15)
	200	22.50±3.02	(25, 25, 24, 22, 22, 17)

*Three rabbits were used for each inoculum.
†Mean±S.D.

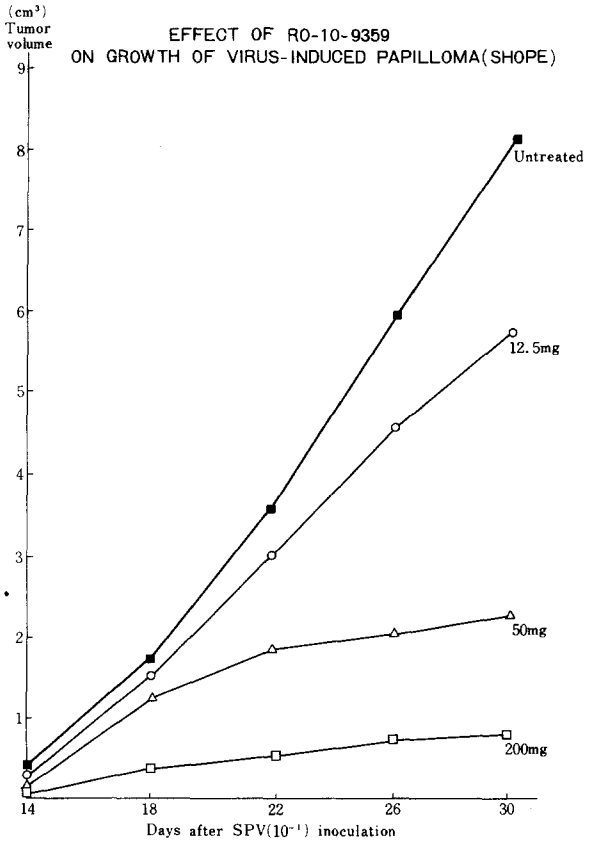

Fig. 1*a*. *Effect of retinoid* (*Ro* 10-9359) *on growth of SPV-induced papilloma. Each point represents mean volume of six typical papillomas in untreated controls and retinoid* (*Ro* 10-9359)*-treated rabbits inoculated with* 10^{-1} *SPV* (1000 ID_{50}). *The retinoid was administered in doses of* 12.5, 50, 200 mg/kg, *respectively, dissolved in peanut oil. The control rabbits were given the vehicle only. The retinoid or control material was given i.m. twice weekly.*

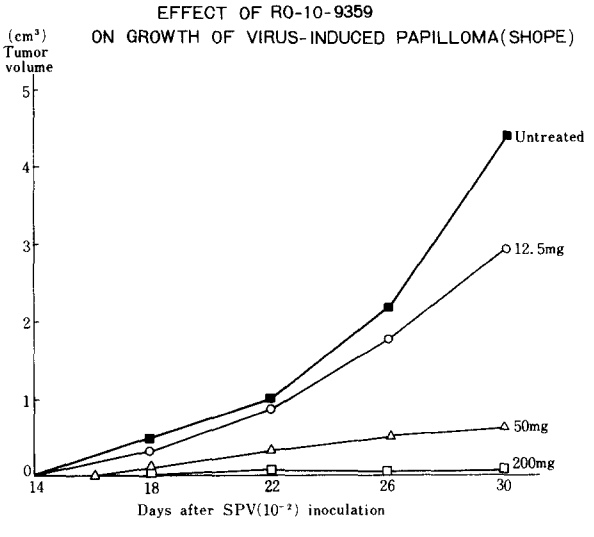

Fig. 1*b*. *Effect of retinoid* (*Ro* 10-9359) *on growth of SPV-induced papilloma. The inoculum was* 10^{-2} *SPV* (100 ID_{50}). *Other details of the experiment were as described in the Fig.* 1*a legend.* (■—■) *Untreated* (*control*) (N=3), (○—○) *treated with* 12.5 mg/kg (N=3); (△—△) *treated with* 50 mg/kg (N=3); (□—□) *treated with* 200 mg/kg (N=3). N: *Number of animals in one group.*

Effect on growth of established papillomas

Twenty-three well-established SPV-induced papillomas were selected and the treatment with the retinoid was given to 12. A relatively high dose of 200 mg/kg of the retinoid was given to the tumor-bearing animals twice weekly. The remaining 11 served as the con-

trol. As can be seen in Table 2, the compound had an inhibitory effect on the growth of the tumors. After 14 days, the tumors in the untreated animals continued to grow prolifically while those in treated animals all showed marked retardation of growth with a reduction in tumor volume.

Table 2. Treatment of virus-induced papilloma (Shope) with a retinoic acid analog (Ro 10-9359)

Treatment	Tumor volume (mm^3) Day 0	Day 14	Change in volume (%)
	1058	6045	+471.3
Untreated	714	3920	+449.0
(control	1863	7752	+316.1
(N=6)	3240	10,296	+217.8
	1008	3200	+217.4
	3750	10,404	+177.4
	5304	14,400	+171.5
	5040	12,312	+144.3
	4050	7616	+ 88.0
	6300	10,923	+ 73.4
	4524	7840	+ 73.3
Treated	5642	3496	−38.1
(200 mg/kg,	1320	630	−52.3
bi-weekly)	3375	1320	−60.9
(N=6)	9282	3496	−62.3
	1026	288	−71.9
	3596	630	−82.5
	5760	760	−86.8
	11,200	1368	−87.8
	6800	627	−90.8
	3672	288	−92.2
	6240	483	−92.3
	6696	256	−96.2

N: Number of rabbits in each group.

SPV-induced papillomas spontaneously regress at a rate of about 30% [12]. All such tumors were excluded from the experiment. Furthermore, the tumors given treatment were mostly those of 4–8 weeks after the SPV-inoculation, as spontaneous regression usually takes place in papillomas within 4 weeks p.i.

Approximately 60% of all tumors in the treated animals completely regressed during the 4–10 weeks after cessation of treatment. Once regression had occurred there was no re-growth.

Histopathological findings

Macroscopically, the tumors affected by the retinoid treatment often have a yellowish, anemic appearance. In the histological observation, the proliferating papillomatous growth (Fig. 2a) begin to show signs of degeneration in the epithelial layer at around 7 days of treatment (Fig. 2b), and this eventually led to destruction of the neoplastic cells and a marked reduction in thickness of the hyperplastic epithelial cell layer characteristic of SPV-induced palillomas by 14 days (Fig. 2c).

Effect on transplantable carcinomas

The effects of a relatively high dose (200 mg/kg) of the retinoid (Ro 10-9359) on the growth of the Vx2 and Vx7 carcinomas are shown in Table 3. Administration of the compound to the tumor-bearing animals had no inhibitory effect on the growth of the two strains of transplantable carcinomas, under the conditions used in this experiment. We observed that in rabbits given the retinoid, there was a significant reduction in number and size of pulmonary metastases which occur in the later stage of tumor development. Follow-up studies of this observation are now underway.

Table 3. Treatment of transplantable carcinomas Vx2 and Vx7 with a retinoic acid analog (Ro 10-9359)

Treatment	Tumor volume (mm^3) Day 0	Day 14	Relative growth rate*
Vx2 untreated	252	3016	11.9
(controls)	320	2808	8.7
(N=2)	352	3024	8.5
	352	2600	7.4
Vx2 treated	672	8370	12.4
(200 mg/kg,	624	7230	11.6
bi-weekly)	576	6300	10.9
(N=2)	684	4992	7.3
Vx7 untreated	110	6050	55.2
(controls)	140	4554	32.5
(N=2)	96	4400	45.8
	144	5775	40.1
Vx7 treated	72	3591	49.8
(200 mg/kg,	72	3168	44.0
bi-weekly)	156	5082	32.6
(N=2)	130	3600	27.7

*Tumor volume at 14 days/tumor volume at 0 day.
N: Number of rabbits in each group.

Assay of retinoids in papillomas and other tissues

The primary aim of the assay was to determine whether the compound administered systemically could actually be detected in the

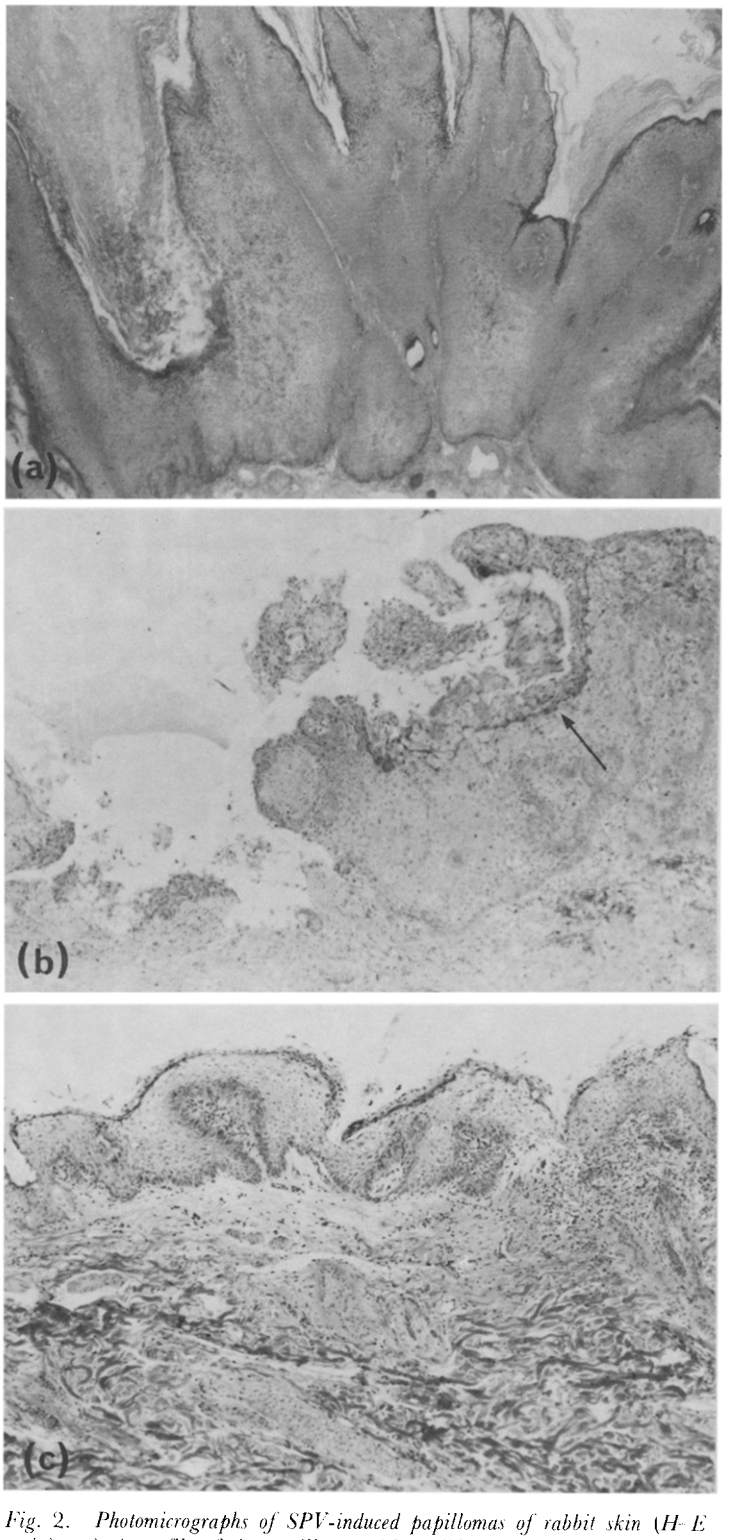

Fig. 2. Photomicrographs of SPV-induced papillomas of rabbit skin (H-E stain). (a) A profile of the papilloma with proliferating growth of epithelial elements, before the treatment (×60). (b) A section of biopsy specimen of papilloma from retinoid (Ro 10-9359)-treated rabbit, 7 days after initiation of the treatment. Note destruction and demarkation in the papillomatous cell layer (arrow) (×120). (c) A specimen from a rabbit treated 14 days with the retinoid. The height of hyperplastic epithelium is remarkably reduced. (×120).

target papilloma tissue. Another point of interest was to see whether the active form of the retinoid, considered to be a compound with a free carboxyl group at the end of the side-chain of the molecule [13] was present. Results of assay for retinoids in various rabbit tissues are shown in Table 4. As expected, both Ro 10-9359 and its acid form, Ro 10-1670, were identified in the SPV-induced papillomas of treated animals. The liver tissues of treated rabbits also contained two forms of the retinoic acid analogs.

The underlaying principle of the suppressive effect of the retinoid (Ro 10-9359) is probably much the same as that of its prototype compounds, retinoic acid, its esters and retinol, all of which are known to exert prophylactic and therapeutic actions on epithelial neoplasia. Our results also support the early findings of McMichael [1].

Recent demonstration of a receptor-like substance of protein nature and specific binding affinity to retinol [14] and retinoic acid [15] has led to the concept that these binding

Table 4. Assay of retinoids in various tissues of retinoid (Ro 10-9359) treated and untreated rabbits

		Content of retinoids (μg/g)†			
Tissue	Treatment*	Ro 10-9359	Ro 10-1670	Retinol	Retinoic acid
Papilloma	200 mg‡	0.05	0.15	0.05	0.02
Papilloma	200 mg	0.15	0.07	0.10	—
Papilloma	200 mg	0.15	0.05	±	—
Papilloma	12.5	—	—	±	—
Papilloma	None	—	—	—	—
Vx2 carcinoma	None	—	—	0.15	—
Vx7 carcinoma	None	—	—	0.14	—
Liver	200 mg	0.66	1.65	8.34	—
Liver	200 mg	0.39	1.56	41.30	—
Liver	None	—	—	75.00	0.75
Blood	200 mg	0.06	0.04	0.09	—
Blood	None	—	—	0.31	—
Skin	None	—	—	0.08	—
Skin	None	—	—	±	—
Kidney	None	—	—	1.50	—
Lung	None	—	—	0.40	—

*With a retinoid (Ro 10-9359).
†Per wt.
‡Per kg, bi-weekly.
(±)=Trace amount.
(−)=Negative or below the sensitivity of the assay.

DISCUSSION

An aromatic analog of retinoic acid, Ro 10-9359, systemically administered to the host animals during the induction and developmental stages of SPV-induced papilloma (Shope) of rabbit skin, exhibited a marked inhibitory effect on growth, and the effect was dose dependent. In the case of well-established papillomas, administration of a relatively large dose (200 mg/kg) of the retinoid drastically diminished the growth and this effect lasted during the ten week observation period. These results agree with those reported by Bollag in chemically-induced epithelial neoplasia of mice [2].

proteins may play an essential role in the mechanism of action of retinoids. We have also found cellular retinoic acid-binding protein (cRABP) in tissue extracts from Shope papillomas [16]. Furthermore, the level of cRABP in rabbit papilloma increased with the developmental stage and formed a peak around 40 days p.i. of SPV. The normal skin extracts showed an extremely low level of cRABP activity. The transplantable carcinomas Vx2 and Vx7 which originated from SPV-induced papillomas over 25 yr ago and both of which were found to be insensitive to the treatment with the retinoid also contained a low level of cRABP.

It is assumed that the active form of the compound is the one with a free carboxyl group at the end of the side chain of the molecule [13]. The present data showing accumulation of the acid form of the retinoid (Ro 10-1670) in the papillomatous tissues of animals treated with Ro 10-9359 tends to support this assumption. The conversion of the compound to the active acid form may well take place in the target epithelial tissue *per se* or in a remote organ or tissue like liver and subsequently be transferred to the tumorous tissue. Ongoing studies are expected to elucidate the mechanism of conversion and further action of the compound.

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