Augmentation of Tumor Immunity in Mice by Intrallesiomal Injection of Vitamin A

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We investigated the antitumor effect of vitamin A (VA) using the double grafted tumor technique to examine whether VA administered into a primary tumor (intrallesiomally or i.l.) accelerates antitumor immune reactions so that growth of the secondary tumor may be more effectively inhibited than by other systemic administration routes. In the double grafted tumor system, where BALB/c mice were inoculated with MethA fibrosarcoma cells into the right inguinal region (1 × 10⁶ cells) on day 0 and later into the left (3 × 10⁶ cells) on day 10, the injection of VA at a dose of 1000 IU/mouse i.l., s.c., i.p., and i.v. on days 3 through 7 inhibited the growth of the secondary tumor to the same extent, while VA at the i.l. dose of 100 IU/mouse into the primary tumor inhibited more effectively than by any other administration route. VA did not inhibit the secondary MethA growth in BALB/c (nu/nu) mice. The spleen cells taken from VA-treated tumor-bearing mice prevented the growth of MethA tumors in naive BALB/c mice when given as a mixture with the MethA inoculum (the Winn assay). The delayed-type hypersensitivity (DTH) response to methylated bovine serum albumin (MBSA) antigen was augmented when VA (1000 IU) was injected at the site of the antigen injection. These results suggest that the direct interaction of VA with the tumor cells may be necessary for the tumor immunity-potentiating effect of VA, and that T-lymphocyte-mediated tumor immunity is involved in the anti-tumor effect of VA. The antitumor mechanism of VA seems to involve retinoid receptors, because the benzoic acid derivative Am80, which has been reported to exert retinoidal activity by binding to specific retinoid receptors, also showed activity.

Key words vitamin A; intrallesiomal injection; tumor immunity; double grafted tumor system; retinoid receptor

The antitumor activity of systemically (p.o. or i.p.) administered vitamin A (VA) and its natural and synthetic analogs (retinoids) is well known,¹⁻⁸ but their mechanism of action appears to be complex.⁹⁻¹¹ A number of studies have shown that VA-mediated antitumor effects are most often observed with a tumor capable of evoking immune responses,¹²⁻¹⁶ and that VA was more effective against allogeneic tumors than against syngeneic tumors.¹⁷ Many authors suggested that the VA-mediated enhancement of tumor immunity was mediated by tumor-specific killer T cells.¹⁸⁻²⁰ Jiang et al. have proposed that retinoids may augment antitumor immunity by potentiating the capacity of IL-2 to induce T-cell proliferation.²¹ However, its effect is also observed in immunodeficient mice,²² suggesting that other mechanisms may also be involved. Retinoids have been shown to modulate the expression of a variety of genes involved in growth²³⁻²⁴ and differentiation.²⁵ They are also known to regulate aberrant differentiation²⁶ and revert malignant cells to normal cells.²⁷⁻²⁹ Thus, it is unclear whether tumor inhibition by VA is the result of a direct effect on tumor cells,³⁰ a potentiated immune response, or a combination of both.³¹⁻³³

We have previously reported a double grafted tumor system in mice where a drug is injected into the primary tumor (intrallesiomally or i.l.) grafted at the right inguinal region, and the mice are later challenged with a secondary, larger, inoculum of the same tumor cells at the left inoculum.³⁴ The direct and indirect antitumor effects of the drug could be evaluated by measuring the growth of both primary and secondary tumors. Thus, we attempted to examine whether VA administered into the primary tumor may accelerate antitumor immune reactions so that growth of the secondary tumor is more effectively inhibited than by other systemic administration routes. We also examined whether VA administered in close proximity to the antigen injection site may augment delayed-type hypersensitivity (DTH), another cell-mediated immune reaction, to methylated bovine serum albumin (MBSA). The resulting data indicated that the direct interaction of VA with the tumor cells seems to be required for its indirect host-mediated antitumor effect, and that growth inhibition of the secondary tumor by VA is exerted by a thymus-dependent T cell-mediated mechanism.

MATERIALS AND METHODS

Chemicals VA miscible retinol palmitate: (1515 IU/mg), (Eisai Co., Ltd., Tokyo), anti-asialo GM1 and anti-Thy antibodies (Wako Pure Chemical Industries Co., Ltd., Osaka), MBSA and ⁵⁻carrageenan (Sigma Chemical Co., St. Louis, MO) were used. Am80, 4-(5, 6, 7, 8-tetrahydro-5, 8, 9-tetramethyl-2-naphthalenyl)carbamoyl) benzoic acid, was synthesized in the Lead Optimization Research Laboratory of this company.

Mice and Tumor Six-week-old male BALB/c and BALB/c (nu/nu) mice were purchased from Charles River Japan (Atsugi, Kanagawa) and maintained on commercial laboratory chow in an air-conditioned room at 23 ± 1°C and 55 ± 5% humidity. MethA, a methylcholanthrene-induced fibrosarcoma, was maintained in BALB/c mice in ascitic form by intraperitoneal inoculation of 10⁵ cells in 0.1 ml physiological saline at 7-d intervals, and colon 26 adenocarcinoma in BALB/c mice in solid form by the subcutaneous inoculation of 10⁵ cells in 0.1 ml Hank’s balanced salt solution (HBSS) at 14-d intervals. The viability of the cells was assessed by the trypsin blue exclusion method.

Double Grafted Tumor System and Drug Treatment The double grafted tumor system described by Ebina and Murata was modified with respect to the inoculation size and timing of the tumor challenge as follows. One million or

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Intraluminal MethA fibrosarcoma cells in 0.1 ml saline were inoculated intracutaneously in the right inguinal region of BALB/c mice on day 0, and 3 × 10^6 MethA cells were inoculated in the left on day 10. VA (1–5000 IU/0.05 ml) or 0.1 ml/mouse) was dissolved in saline and administered i.l. in the right inguinal region or by other routes on days 3 through 7. The size of both primary and secondary tumors was measured with a caliper on days 10, 17 and 24, and expressed as the mean of the long and short diameters (mm). The difference in tumor size between the control and experimental groups was statistically analyzed using Student’s t test.

**Effect on the Secondary Tumor Growth after Excision of the Primary Tumor** The experimental method is essentially the same as the preceding double-grafted tumor system, except that the primary inoculum was injected into the right footpad.34 One million MethA cells in 50 μl saline were inoculated into the right footpad of BALB/c mice on day 0. VA (1000 IU, 660 μg/50 μl/mouse) was injected into the right footpad on days 3 through 7. On day 10, when the local growth reached 6–8 mm in thickness, the leg with the primary tumor was amputated after ligation above the knee joint, and the mouse was then challenged with MethA (3 × 10^6) cells into the left inguinal region half an hour after tumor resection. Growth of the secondary tumor was observed for the following 14 d to evaluate the development of sineaomatin immunity.

**Preparation of Spleen Cells and Tumor-Neutralization Test (the Winn Assay)** The tumor-neutralization test was carried out according to the Winn assay, and the preparation of splenocytes was the same as described before.38

**DTH Response to MBSA** An aliquot (100 μl) of 0.25% MBSA in saline was subcutaneously injected in the right inguinal region of BALB/c mice on day 0. Ten days later, the mice were challenged by subcutaneously injecting 50 μl of 0.25% MBSA solution into the right hind paw, and saline was injected into the left as a control. Twenty-four hours after the challenge, DTH response was estimated by measuring footpad swelling with a dial thickness gauge (G-2, Ozaki Seisakusyo) and was expressed as the difference in thickness (mm) between the right and left footpads.39 VA (1000 IU/50 μl/mouse) was administered subcutaneously into the right inguinal region at the site of antigen injection, or in the left inguinal region on day 0, 2 h after immunization on day 0 or 2 h after the challenge on day 11.

**RESULTS**

**Dose Response of the Inhibitory Effect of the Intraleisional Administration of VA on the Growth of Both Primary and Secondary Tumors** When mice were primed with 10^6 cells of MethA into the right inguinal region on day 0 and the secondary inoculum was injected to the left on day 10 in the double-grafted tumor system, an inoculum size of 3 × 10^6 or more cells was necessary to produce the same rate of tumor growth as that of the primary tumor (data not shown). This indicates that the MethA-bearing mice acquired partial resistance to MethA cells. Therefore, we chose the inoculum size of 3 × 10^6 cells for the secondary tumor in the present study. An intraleisional injection of VA at the dose of 100 IU or more per animal on days 3 through 7 markedly inhibited the growth of the secondary tumor in this double-grafted tumor system, whereas growth of the primary tumor was only slightly inhibited at the highest doses of 1000 and 5000 IU/mouse of VA (Fig. 1).

Am80, a benzoic acid derivative which has been shown to interact with retinoid receptors,30 was also effective, but its dose-dependency curve was U-shaped, with a maximal effect at the dose of 2.4 mg/kg (Table 1).

When colon 26, which is antigenically unrelated to MethA, was inoculated as the secondary inoculum (3 × 10^6 cells), its growth was not affected at all by VA (1000 IU/mouse) injected intraleisionally into the primary MethA tumor (data not shown).

**Effect of the Route of VA Administration on the Growth of the Secondary Tumor** Other parenteral routes (i.p., i.v., and s.c. in the middle of the abdomen) were also effective when VA was injected at the dose of 1000 IU, but the p.o. route was not effective (Table 2). At the dose of 100 IU, however, only the i.l. route showed efficacy, while the other injection routes (i.p., s.c., i.v. and p.o.) showed no effect. We then examined whether regional lymph nodes are involved in the VA-mediated inhibition of secondary tumor growth. An

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**Table 1. Effect of Intraleisional Injection of Am80 on the Growth of MethA in the Double Grafted Tumor System**

<table>
<thead>
<tr>
<th>(mg/kg)</th>
<th>Tumor size on day 24 (Diameter mm ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primary</td>
</tr>
<tr>
<td>Control</td>
<td>29.8 ± 0.8</td>
</tr>
<tr>
<td>VA (33.3)</td>
<td>26.6 ± 0.4**</td>
</tr>
<tr>
<td>Am80 (0.8)</td>
<td>27.3 ± 0.7*</td>
</tr>
<tr>
<td>(2.4)</td>
<td>13.9 ± 1.1**</td>
</tr>
<tr>
<td>(8)</td>
<td>19.1 ± 0.8**</td>
</tr>
<tr>
<td>(24)</td>
<td>23.5 ± 0.8**</td>
</tr>
</tbody>
</table>

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MethA cells (1 × 10^6) in 0.1 ml saline were inoculated intradermally into the right inguinal region of BALB/c mice on day 0. Drug or saline was administered intraleisionally on days 3 through 7. On day 10, 3 × 10^6 MethA cells were inoculated intradermally into the left inguinal region. The solid and dashed lines represent the growth of the primary and secondary tumors, respectively. Each point indicates the mean tumor diameter (N = 8). Statistical significance of difference from the control, *p < 0.05, **p < 0.01, ***p < 0.001 (Student’s t test).
intrallesional (right groin) injection of VA at the dose of 100 IU markedly inhibited the growth of the secondary tumor (Table 3, A vs. B, p<0.01), while VA injection (s.c.) into the right footpad slightly inhibited the secondary tumor, but the difference from the control was statistically insignificant (A vs. C). On the other hand, VA injection into the left footpad did not at all inhibit the secondary tumor. The inhibition by i.l. injection of VA was significantly greater than by ipsilateral s.c. injection (B vs. C, p<0.01).

**Relationship between the Inoculum Size of the Primary Tumor and the Antitumor Activity of VA**

The relationship between the primary tumor inoculum size and the antitumor activity of VA was then investigated (Fig. 2). When 1<sub>x</sub>10<sup>6</sup> or fewer cells were used as the primary inoculum, the primary tumor did not take; 5<sub>x</sub>10<sup>6</sup> cells or more were necessary to generate the primary tumor. When mice were inoculated with 5<sub>x</sub>10<sup>5</sup> or 1<sub>x</sub>10<sup>6</sup> cells, i.l. administration of VA at the dose of 1000 IU/mouse/d on days 3 through 7 suppressed not only the secondary but also the primary tumor, while with 3<sub>x</sub>10<sup>5</sup> or 1<sub>x</sub>10<sup>5</sup> cells VA suppressed only the secondary tumor. When the inoculate was less than 10<sup>5</sup> cells,

### Table 2. Effect of Various Routes of VA Injection on Growth of the Secondary Tumor

<table>
<thead>
<tr>
<th>Routes</th>
<th>Secondary tumor size on day 24 (Diameter mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VA (100 IU)</td>
</tr>
<tr>
<td>Control</td>
<td>14.7±0.7</td>
</tr>
<tr>
<td>i.l.</td>
<td>2.6±1.6***</td>
</tr>
<tr>
<td>s.c.</td>
<td>15.1±0.4</td>
</tr>
<tr>
<td>i.p.</td>
<td>14.4±0.6</td>
</tr>
<tr>
<td>i.v.</td>
<td>13.6±0.8</td>
</tr>
<tr>
<td>p.o.</td>
<td>15.1±0.5</td>
</tr>
</tbody>
</table>

MethA cells (1<sub>x</sub>10<sup>5</sup>) in 0.1 ml saline were inoculated intradermally into the right inguinal region of BALB/c mice on day 0. VA (1000 or 100 IU) or saline was administered in various routes on days 3 through 7. On day 10, 3<sub>x</sub>10<sup>5</sup> MethA cells were inoculated into the left inguinal region. Significant difference from the control group, ***p<0.001 (Student's t test). N=7.

### Table 3. Effect of VA Injected Intralesionally or Subcutaneously into the Footpad on Growth of the Secondary Tumor

<table>
<thead>
<tr>
<th>Mice</th>
<th>Right tumor (Day 24)</th>
<th>Left tumor (Day 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diameter (mm)</td>
<td>Diameter (mm)</td>
</tr>
<tr>
<td>(A) Control</td>
<td>28.4±0.4</td>
<td>13.3±0.2</td>
</tr>
<tr>
<td>(B) VA</td>
<td>26.3±0.4</td>
<td>3.2±1.0</td>
</tr>
<tr>
<td>Intralesimal</td>
<td>27.8±0.4</td>
<td>11.0±1.2</td>
</tr>
<tr>
<td>(C) VA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right footpad</td>
<td>28.0±0.5</td>
<td>12.2±0.7</td>
</tr>
<tr>
<td>(D) VA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left footpad</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MethA cells (1<sub>x</sub>10<sup>5</sup>) in 0.1 ml saline were inoculated intradermally into the right inguinal region of BALB/c mice on day 0. VA (100 IU) in a volume of 0.05 ml was administered intradermally (B) or subcutaneously into the right footpad (C) or left footpad (D) on days 3 through 7, and saline (0.05 ml) was injected into the right inguinal region as a control (A). On day 10, 3<sub>x</sub>10<sup>5</sup> MethA cells were inoculated intradermally into the left inguinal region. Statistical significance of difference in the left (secondary) tumor size was analyzed by the Tukey-Kramer method, A vs. B: p<0.01, A vs. C: NS, B vs. C: p<0.01, C vs. D: NS.

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**Fig. 2. Effect of Intrallesional VA Injection on the Growth of MethA Fibrosarcoma in Mice Inoculated with Various Numbers of Primary Tumor Cells**

Various numbers of MethA cells (0<sub>x</sub>1<sub>x</sub>10<sup>5</sup>) were inoculated intradermally into the right inguinal region of BALB/c mice on day 0. VA (1000 IU/mouse; ●) or saline (○) was administered intradermally at the site of inoculation or subcutaneously, in the case of no tumor growth, on days 3 through 7. On day 10, 3<sub>x</sub>10<sup>5</sup> MethA cells were inoculated intradermally into the left inguinal region. The solid and dashed lines represent the growth of the primary and secondary tumors, respectively. Each point indicates the mean tumor diameter (N=6). Statistical significance of difference from the control, * p<0.05, ** p<0.01, *** p<0.001 (Student's t test).
Table 4. Effect of Intrallesional (Right Paw) Injection of VA on the Growth of the Reinoculated MethA Tumor

<table>
<thead>
<tr>
<th>Mice</th>
<th>Right tumor thickness (mm)</th>
<th>Left tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 10</td>
<td>Day 24</td>
</tr>
<tr>
<td>(A) Non-amputated</td>
<td>Control 7.1±0.2</td>
<td>14.6±0.4</td>
</tr>
<tr>
<td></td>
<td>VA 6.2±0.1***</td>
<td>14.7±0.4</td>
</tr>
<tr>
<td>(B) Right-leg amputated</td>
<td>Control 7.1±0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VA 6.2±0.1***</td>
<td></td>
</tr>
<tr>
<td>(C) Left-leg amputated</td>
<td>Control 7.1±0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VA 7.1±0.1</td>
<td></td>
</tr>
</tbody>
</table>

MethA cells (1×10^6) cells/0.05 ml) were inoculated into the right hind paw on day 0. VA (100 U/0.05 ml) was injected into the right paw (intrallesionally) on days 3 through 7. On day 10, when control tumor thickness reaches about 7 mm, mice were divided into three groups: Group A: The primary tumor was allowed to grow. Group B: The tumor-bearing leg (right) was amputated. Group C: The tumor-free leg (left) was amputated. Half hour after the amputation, all mice were challenged with MethA (3×10^6 cells/0.1 ml) into the left inguinal region on day 10. Significant difference from the control group, **p<0.01, ***p<0.001 (Student’s t-test). N=8.

Table 5. Effect of Timing of VA Administration on Growth of MethA Tumors

<table>
<thead>
<tr>
<th>VA administration</th>
<th>Tumor size on day 29 (Diameter mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primary 24.6±1.2</td>
</tr>
<tr>
<td></td>
<td>Day 1, 2 21.8±1.4</td>
</tr>
<tr>
<td></td>
<td>Day 5, 6 22.1±3.9</td>
</tr>
<tr>
<td></td>
<td>Day 8, 9 23.9±1.1</td>
</tr>
<tr>
<td></td>
<td>Day 12, 13 25.9±0.8</td>
</tr>
<tr>
<td></td>
<td>Day 15, 16 26.3±1.2</td>
</tr>
</tbody>
</table>

MethA cells (1×10^6) in 0.1 ml saline were inoculated intradermally into the right inguinal region of BALB/c mice on day 0. VA (1000 U/mouse) or saline (C) was administered intradermally according to various time schedules. On day 14, 3×10^6 MethA cells were inoculated intradermally into the left inguinal region. Significant difference from the control group, *p<0.05, **p<0.01 (Student’s t-test). N=8.

subcutaneously administered VA did not inhibit the growth of the secondary tumor. These results indicate that the primary tumor take or growth is necessary for VA to inhibit growth of the challenging tumor, and that VA exerts its antitumor action through a host-mediated effect by acting directly on the tumor cells and/or by immunocytes interacting with the tumor cells.

Effect of Tumor Excision on the Antitumor Effect of VA in the Double Grafted Tumor System It is known that the growth of a secondary tumor is sometimes inhibited simply by the presence of an earlier inoculated, growing tumor. We examined whether VA was able to inhibit the growth of the secondary tumor after surgical removal of the primary tumor. To facilitate the surgical removal of the primary tumor, we inoculated tumor cells into the footpad. After the mice were challenged with MethA cells into the left inguinal legion on day 10, the mice were divided into 3 groups (Table 4, A, B, C). The mice of the first group were left intact as non-amputated tumor-bearing controls. In the second group, the right leg bearing the primary tumor was amputated after ligation above the knee joint. In the third group, the tumor-free left leg was amputated. Intralesionally administered VA did not affect the growth of the primary tumor, while it inhibited the growth of the secondary tumor in the intact group (A). The degree of inhibition of the secondary tumor was 84.3%, and 12 mice out of the 15 challenged mice were tumor-free. In contrast, the group in which the tumor had been excised (B) showed much less inhibition (27.9%) of the secondary tumor growth and produced no tumor-free mice. In the tumor-bearing mice with the left leg amputated (C), growth of the secondary tumor was inhibited almost to the same extent (79.6%) as in the intact group (A), and the VA treatment produced 12 tumor-free mice. These results indicate that the presence of a primary tumor is necessary for the optimal antitumor effect of VA, and the attenuation of the VA action following tumor excision did not result from surgical stress.

Optimal Timing of VA Administration for Its Inhibitory Effect on MethA Growth When VA was i.d. administered for two consecutive days, VA was most effective in inhibiting the secondary growth (secondary inoculation on day 14) when administered on days 5 and 6 or days 8 and 9, but showed no effect when given on days 1 and 2 or on days 15 and 16 (Table 5), indicating that there is an optimal period (days 5 through 9) for administration during the induction phase of tumor immunity during which VA exerts its antitumor effect.

Effect of Intraperitoneal Treatment with Anti-Thy-1 and Anti-asialo GM1-Antibodies, and χ-Carrageenan on the
Antitumor Activity of VA  To investigate the type of effector cells involved in VA-induced tumor immunity, MethA-primed and VA-treated mice were challenged with secondary tumor cells on day 13, then injected intraperitoneally with anti-Thy or anti-asiatio GM1 antibodies or λ-carrageenan on days 13, 14, 17, and 20 (Fig. 3). It was reported that NK cells and macrophages were depleted in vivo by treatment with 20 μl/mouse of anti-asiatio GM1 and 1.5 mg/mouse of λ-carrageenan, respectively. The treatment with the anti-Thy antibody completely abolished the tumor-inhibitory activity of VA, while neither anti-asiatio GM1 antibody nor λ-carrageenan interfered with the antitumor action of VA.

Effect of VA on the Growth of MethA Cells in BALB/c (nu/nu) Mice  We then examined whether the tumor-inhibiting activity of VA is thymus-dependent by using BALB/c (nu/nu) mice in our double grafted tumor system. Under the conditions in which VA markedly inhibited the secondary tumor growth in BALB/c mice, the vitamin failed to show its antitumor effect in age-matched BALB/c (nu/nu) mice (data not shown).

Tumor-Neutralizing Activity of the Spleen Cells Obtained from the Tumor-Bearing VA-Treated Mice (the Winn Assay)  Involvement of spleen cells in the VA-induced potentiation of tumor immunity was tested using the Winn assay. When 1 × 10⁶ MethA cells were admixed with 1.5 × 10⁷ spleen cells taken from the tumor-bearing mice treated with VA, then inoculated intracutaneously at the right inguinal region of BALB/c (Fig. 4A) or BALB/c (nu/nu) recipient mice (Fig. 4B), there was no or only slight tumor growth, respectively. In contrast, MethA cells admixed with splenocytes from non-treated tumor-bearing mice grew. Tumor-free mice after the Winn assay were challenged with 3 × 10⁶ MethA cells to examine whether they were immune to MethA cells. All mice were resistant to the challenge with MethA cells, showing long-lasting tumor-specific immunity (data not shown).

Adjuvant Effect of VA on DTH Response to MBSA Antigen in Mice  We examined whether VA was able to augment the DTH response to MBSA antigen. Augmentation of the DTH response was observed on day 12 (the day after the challenge on day 11), when VA (1000 IU/50 μl) was injected into the right inguinal region (at the site of antigen injection) 2 h after immunization, but not when VA was injected into the left inguinal region (Fig. 5). VA injected into the right or left inguinal region at the time of challenge (day 11) showed no effect. This suggests that exposure of the immunization site to VA in the induction phase is essential for the DTH-augmenting action of the vitamin.

DISCUSSION  We have previously reported that intralesionally administered 6-mercaptopurine (6-MP) showed antitumor activity by potentiating tumor immunity rather than by cytotoxic carcinostasis in the double grafted tumor system in mice. We showed in the present investigation, using the same technique, that intralesionally administered VA also has a similar enhancing effect on tumor immunity. Since VA was most effective in inhibiting the secondary tumor growth when given
on days 5 and 6, or days 8 and 9 (Table 5), but showed no effect when given right after the primary or secondary inoculation. VA must enhance the late induction phase rather than the elicitation phase of the immune response, although the precise step involved remains to be elucidated. Furthermore, the optimal enhancement of tumor immunity by VA required the presence of a substantial amount of growing tumor cells as the primary tumor (Fig. 2). Also, the VA enhancement of antitumor immunity was tumor-specific, because VA did not inhibit the growth of colon 26 in Meth-A-primed mice.

No VA-mediated enhancement of tumor immunity was observed in the T cell deficient BALB/c (nu/nu) mice. In fact, the anti-tumor activity of VA was abolished by treatment with an anti-thymus antibody (Fig. 3). The observation that the spleen cells obtained from VA-treated tumor-bearing euthymic mice, when mixed with Meth A cells before inoculation, prevented the growth of the tumor in BALB/c and BALB/c (nu/nu) mice (Fig. 4) could be taken as another piece of evidence that T cells are involved in the tumor immunity-enhancing action of VA. Dennert et al., Lotan et al., Malkovsky et al., and Tomita et al. have also reported the VA-induced enhancement of tumor immunity, and all these authors suggested the involvement of tumor-specific killer T cells. In our study, NK cells and macrophages did not appear to be significantly involved, because anti-asialo GM1 antibody and carrageenan did not affect the antitumor activity of VA (Fig. 3).

The most noteworthy finding in the present study is that the i.l. route of VA administration is the most effective for the tumor immunity-enhancing effect of VA (Table 2). A number of groups have attempted the intraleisional injection of antitumor agents to eradicate tumors in animal models. Examples of this include the intraleisional injection of cytotoxic/cytostatic low molecular weight drugs to give high local concentrations, Bacillus Calmette-Guerin (BCG) to evoke local inflammation, interleukin 1b (IL-1b) to cause the in situ infiltration of effector cells, and IL-2 to bring out systemic tumor immunity. However, few basic studies have investigated intraleisional treatment with VA in animal models. Felix et al. reported that the intraleisional injection of VA destroyed the superficial structure of the tumor by causing inflammatory necrosis. Levine also reported that nonspecific inflammation at the site of VA injection was responsible for tumor cell necrosis. It is not clear why the intraleisional injection of VA inhibits the growth of the secondary tumor more effectively than by any other administration route. VA has been shown to potentiate a DTH reaction to sheep red blood cell (SRBC) only when it was subcutaneously administered at the time and site of immunization with the antigen. Our present study also demonstrated a similar potentiation of DTH to MBSSA by VA administered at the site of antigen injection (Fig. 5).

It has been reported that the local excision of lymph nodes prevents the host from eradicating tumor cells and that the draining lymph node plays an important role when VA exerts its adjuvant action. In our study, the intraleisional injection of VA inhibited the secondary tumor growth much more strongly than the subcutaneous injection of VA into the ipsilateral footpad. The contralateral s.c. injection had little effect on the growth of the secondary tumor (Table 3). These results suggest that VA administration in close proximity to the tumor tissue is prerequisite for its immunological antitumor action.

Fisher et al. proposed two types of tumor resistance: the resistance to a second inoculation in the presence of a growing primary tumor, named concomitant immunity, and the resistance remaining after excision of the primary tumor, named sinecomitant immunity. VA appeared to potentiate both types of immunity because excision of the primary tumor largely, but not completely, abolished the VA action (Table 4).

Shudo et al. reported that a benzoic acid derivative, Am80, exerted retinoid activity by binding to certain specific retinoid receptors. In our study, 2.4–8 mg/kg/d of Am80 (equimolar to VA 3.3–10 mg/kg), given intraleesionally on days 3 through 7, inhibited the secondary tumor growth in the double grafted tumor system (Table 1). Thus, one of the retinoid receptors is likely to be involved in the VA (as well as Am80)-mediated enhancement of tumor immnunity.

In conclusion, we herein present clear evidence that the intraleisional injection of VA induces strong tumor immunity and helps prevent the growth of a secondary tumor. The anti-tumor effect of VA may result from its direct interaction with tumor cells, and seems to be elicited through retinoid receptors. Since these studies have been carried out in mice, the clinical relevance of these experimental results is unknown, but intraleisional treatment with VA for a certain period could be recommended before the surgical removal of the primary tumor.

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