Inhibition of Moloney Murine Lymphoma and Sarcoma Growth in Vivo by Dietary Retinoids

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ABSTRACT

The effects of dietary retinoids on the growth of Moloney lymphoma (LSTRA) and sarcoma (MSC) in BALB/c mice were evaluated. Transplantable syngeneic Moloney lymphoma and sarcoma tumors are immunogenic. Preimmunization with LSTRA cells provides protection against subsequent challenge and sarcomas spontaneously regress following injection of an appropriate inoculum of MSC cells. In normal mice fed varying concentrations of all-trans-retinoic acid (RA) and given injections of 10^2 LSTRA cells, RA caused a dose-dependent increase in the number of survivors; 50% of the mice fed RA at 50 mg/kg of diet were long-term survivors. All animals died that were fed a control diet and challenged with 10^3 LSTRA cells. Athymic (nu/nu) mice fed RA were not protected against lymphoma growth, whereas euthymic (nu/+ ) mice were; therefore, the antitumor effect of RA was thymus dependent. Primary immunization with irradiated LSTRA in the presence of RA caused a significant increase in cell-mediated cytotoxicity by spleen cells at 4 days after immunization. However, challenge of animals preimmunized with LSTRA in the presence of dietary RA revealed a dose-dependent inhibition of memory. A significant reduction in MSC growth was also observed in normal mice fed 13-cis-retinoic acid (cRA). A comparison of the primary antilymphoma effect of dietary RA, cRA, N-(all-trans-retinyl)DL-lysine (RL), and N-(4-hydroxyphenyl)retinamide (4-HPR) revealed an efficacy hierarchy of RL > RA > cRA > 4-HPR with RL producing 70% long-term survivors at 115 days after challenge with 10^3 LSTRA cells. These studies indicate that retinoids can inhibit the growth of transplantable, retroviral-induced, immunogenic tumors by thymus-dependent mechanisms and that a newly synthesized retinolaminic acid (RL) is more potent than RA at inhibiting Moloney lymphoma growth.

INTRODUCTION

Vitamin A (retinol) and its analogues (retinoids) are important in normal growth, vision, reproduction, and epithelial differentiation. Early observations (1, 2) that vitamin A-deficient rodents had an increased incidence of papillomas and squamous cell carcinomas resulted in studies of the possible antineoplastic effects of increased dietary vitamin A. Retinoids have been shown to prevent the occurrence and growth of epithelial and mesenchymal tumors induced by chemicals, irradiation, or viruses in experimental animals (3–5). These observed antineoplastic effects of retinoids could be due either to their antipromotion activity (6), to potentiation of antitumor immune responses (7-10), or to a combination of both. The hypothesis that retinoids can potently induce antitumor immune response is supported by several reports demonstrating that retinoid-mediated inhibition of transplantable tumor growth is an increase in cellular immune functions that parallel the growth inhibition of three transplantable tumors (11).

The immunological mechanisms by which retinoids inhibit tumor growth may include increasing natural killer cell activity (11, 12), increasing macrophage tumoricidal activity (14, 15), and stimulating of T-cell-mediated cytotoxicity (16, 17). A recent report has shown that dietary retinyl palmitate causes an increase in cellular immune functions that parallel the growth inhibition of three transplantable tumors (18).

The purpose of the present study was to evaluate the effects of dietary retinoids on the growth of retroviral-induced transplantable tumors in mice and to determine whether thymus-dependent mechanisms are involved. In addition, the effects of RA on immunological memory of mice immunized against a murine lymphoma were evaluated.

The Moloney sarcoma or lymphoma models provide convenient systems to study the effects of modulation of immune function on the growth of antigenic tumors with known virally specified antigens. The Moloney murine sarcoma/leukemia virus complex consists of a replication-defective murine sarcoma virus (M-MuSV) and a murine leukemia virus (M-MuLV) that is required as a helper virus for M-MuSV replication (19, 20). M-MuSV contains a host cellular oncogene (v-mos) responsible for its transforming properties (21) while M-MuLV provides the antigens against which murine hosts respond (22). Mice given injections of an appropriate inoculum of M-MuSV or M-MuSV-transformed cells (MSC) develop tumors that spontaneously regress (23). MSC is a sarcoma cell line of BALB/c origin induced by M-MuSV (24). Inoculation (i.m.) in the thigh of a mouse with 10^5 MSC cells causes regressing tumors, whereas injection of 10^6 cells results in progressing tumors (23). In vivo studies have shown that both thymus-dependent, cell-mediated immune mechanisms (23, 25) and humoral antibodies (26–28) play an important role in tumor regression. In vitro analyses have demonstrated specific cell-mediated cytotoxicity (29, 30) and antibody (31, 32) of both IgG and IgM classes in response to M-MuSV infection. M-MuLV induces lymphomas when inoculated into neonatal mice (33). Even though Moloney sarcomas and lymphomas share antigens specified by M-MuLV (34), spontaneous regression in previously unimmunized animals is not observed following inoculation of syngeneic Moloney lymphoma cells (35). Moloney lymphomas are, however, immunogenic. Appropriate preimmunization provides protection from challenge with viable lymphoma cells (35, 36).

In the experiments of the current study, we have used the LSTRA cell line which is a BALB/c lymphoma cell line induced by M-MuLV (33). Mice given injections i.p. of 10^7 LSTRA cells die with acutic lymphoma within 20–40 days.

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1 The abbreviations used are: RA, all-trans-retinoic acid; RL, N-(all-trans-retinyl)DL-lysine; cRA, 13-cis-retinoic acid; 4-HPR, N-(4-hydroxyphenyl)retinamide; PBS, phosphate-buffered saline; dTdh, thymidine; E:T ratio, effector:target cell ratio.

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cant inhibition of tumor cell growth resulting in long-term survivors following challenge with a tumor inoculum that killed all the animals on a control diet. The tumor growth inhibition was thymus dependent. Also a newly synthesized retinoylamino acid appears to be more effective at inhibiting Moloney lymphoma growth than three other retinoids tested in this study. In addition, dietary RA caused a dose-dependent decrease in immunological memory to Moloney murine lymphoma cells.

MATERIALS AND METHODS

Animals. Pathogen-free female BALB/c mice, both athymic (nu/nu) and euthymic (nu/+), were obtained from the National Cancer Institute (Frederick, MD) and were housed in a barrier-maintained room kept at 23°C with a 12-h light, 12-h dark cycle. Athymic (nu/nu) mice were kept in cages with filter tops. Mice were randomly divided into groups of 5 to 10 mice per group and were given a standard rodent chow (Agway Prolab 1000, Syracuse, NY) and water ad libitum until receiving experimental diets. Seven to 8-week-old mice were used in all experiments.

Diet. All-trans-retinoic acid, 13-cis-retinoic acid, all-trans-N-(4-hydroxyphenyl)retinamide, and N-(all-trans-retinoyl)-DL-leucine (Fig. 1) were synthesized and characterized as previously described (37, 38), and were kept under a nitrogen atmosphere in amber vials at -20°C until used. Retinoids were weighed, added to triptanoin-ethanol vehicle, and mixed into diet (Wayne Feed) at 150.0, 50.0, 25.0, and 12.5 mg/kg of diet. Based on an average consumption of 4 g of feed a day by a 20-g mouse, these diet concentrations correspond to a retinoid consumption of 30.0, 10.0, 5.0, and 2.5 mg/kg body weight/day. Diets were kept in the dark at 4°C and were used within 2 weeks after preparation. High pressure liquid chromatographic analysis of diets for retinoid content indicated that retinoid concentrations remained stable during the duration of the experiments. Retinoids were administered to mice only via their diet.

Tumor Cells and Transplantation. Tumor cell lines were maintained in tissue culture (5% CO₂, 37°C) with RPMI 1640 (Flow Laboratories, McLean, VA) containing 10% heat-inactivated fetal calf serum (Flow), 100 µg/ml gentamycin (Shering Corp., Kenilworth, NJ), and 1% L-glutamine. Monolayers of MSC cells were treated with 0.25% trypsin, washed twice in PBS, pH 7.5, counted, and resuspended in PBS. LSTRA cells were washed twice in PBS, counted and resuspended in PBS. Viability was always >95% as determined by trypan blue exclusion. Mice were given injections either i.p. of 10³ to 10⁶ LSTRA cells or i.m. in the right thigh with 10⁵ MSC cells.

LSTRA Immunization. In the immunization experiments, LSTRA cells were irradiated with 5000 rads from a 137Cs source (Gamma Cell 40, Atomic Energy of Canada, Ltd., Kanata, Ontario, Canada), washed twice in PBS, pH 7.5, counted, and resuspended in PBS. LSTRA cells were washed twice in PBS, counted and resuspended in PBS. Viability was always >95% as determined by trypan blue exclusion. Mice were given injections either i.p. of 10³ to 10⁶ LSTRA cells or i.m. in the right thigh with 10⁵ MSC cells.

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In Vivo Effect of Retinoids on MSC and LSTRA Growth. Groups of 10 BALB/c mice each were fed a control diet or a diet containing retinoid (RA, cRA, RL, or 4-HPR) at 150, 50, or 12.5 mg/kg of diet for 2 weeks, then for the remainder of the experiment they were given a standard rodent chow (Agway Prolab 1000) ad libitum. After 2 weeks of retinoid diet, mice were inoculated i.p. with 10⁵ LSTRA in 0.1 ml PBS. Control animals received 0.1 ml PBS. Mice were observed daily for 70 days to determine time of death. All surviving mice were killed 115 days after LSTRA challenge.

Another experiment evaluated the effect of one retinoid (cRA) on Moloney sarcoma growth. BALB/c mice (6/group) were fed either 50 mg/kg cRA or a control diet for 2 weeks, then inoculated with 10⁵ MSC in the right thigh. Tumor growth in MSC-injected mice was monitored by measuring thigh diameters. Geometric mean increases in thigh diameter were calculated at each time point by using 0.05 mm for no increase.

Cytotoxicity Assay. Splenocytes from three mice per group fed either RA at 50 mg/kg of diet or a control diet for 2 weeks and then inoculating with 5 x 10⁵ LSTRA cells. Mice were observed for survival times.

In Vivo Effects of RA on MSC and LSTRA Growth. MSC or LSTRA cells (10⁴) were cultured at 37°C and 5% CO₂ in triplicate wells of a 96-well flat-bottomed plate (Costar) for 2 to 5 days with varying concentrations of RA (10⁻¹⁰ to 10⁻⁶ M). Twenty-four h prior to termination of the culture, 1 µCi of tritiated thymidine [³H]dThd, 2 Ci/mmol, (Amersham International, Amersham, United Kingdom), was added to each well. The cells were harvested and the [³H]dThd incorporation was measured with a liquid scintillation counter (Beckman, Fullerton, CA).

The involvement of T-cells in retinoid-induced antitumor activity was determined by feeding euthymic (nu/+) and athymic (nu/nu) mice either RA at 25 mg/kg or a control diet for 2 weeks and then inoculating mice with 5 x 10⁵ LSTRA cells. Mice were observed for survival times.

In Vivo Effects of RA on MSC and LSTRA Growth. MSC or LSTRA cells (10⁴) were cultured at 37°C and 5% CO₂ in triplicate wells of a 96-well flat-bottomed plate (Costar) for 2 to 5 days with varying concentrations of RA (10⁻¹⁰ to 10⁻⁶ M). Twenty-four h prior to termination of the culture, 1 µCi of tritiated thymidine [³H]dThd, 2 Ci/mmol, (Amersham International, Amersham, United Kingdom), was added to each well. The cells were harvested and the [³H]dThd incorporation was measured with a liquid scintillation counter (Beckman, Fullerton, CA).

Cytotoxicity Assay. Splenocytes from three mice per group fed either RA at 50 mg/kg of diet or a control diet for 2 weeks and then inoculating with 10⁶ irradiated (5000 rad) LSTRA cells were removed and made into single cell suspensions as previously described (39). Mice were killed on postinoculation day 4. Splenocytes (effectors) were adjusted to different cell concentrations depending on E:T ratio. LSTRA target cells were labeled with [⁵¹Cr], adjusted to 2 x 10⁶/ml, and aliquoted in triplicate at 2 x 10⁴ cells/tube in 0.1 ml together with 0.1 ml of spleen cells at E:T ratios of 50:1, 25:1, and 12.5:1. Controls consisted of spleen cells from unimmunized mice fed either RA at 50 mg/kg or a
normal diet. After a 4-h incubation, the tubes were centrifuged and the pellets and supernatants were assayed for \(^{51}\)Cr activity in a gamma counter. Spontaneous lysis was determined by using spleen cells from nonimmunized mice. Percentage of lysis was calculated according to the following formula:

\[
\% \text{ of lysis} = \frac{\text{Experimental cpm} - \text{spontaneous cpm}}{\text{Maximum cpm} - \text{spontaneous cpm}} \times 100.
\]

**RA Effects on LSTRA Immunization.** Euthymic BALB/c mice were divided into groups that received either a control diet, or diets containing 50 or 12.5 mg/kg RA. The groups were fed either 25 mg of RA/kg of diet or a control diet for 2 weeks and challenged with \(5 \times 10^7\) LSTRA cells. All athymic animals fed RA or control diet died at a similar rate by 30 days after injection of tumor cells. All the euthymic mice on a control diet died within 60 days. However, the group of euthymic animals fed RA had 40% survivors at 70 days post-LSTRA challenge (Fig. 4).

Another experiment was performed to evaluate the effect of a single retinoid, cRA, on the growth of a transplantable sarcoma which expresses similar tumor antigens as LSTRA cells. Injection of moloney sarcoma cell line, MSC, provided a convenient model to assess daily effects of retinoid on tumor growth. Mice fed cRA at 50 mg/kg for 2 weeks and given injections of \(10^5\) MSC cells had a significant reduction in tumor burden in comparison to mice fed a control diet (Fig. 5). On day 11, thigh diameters (logio mean ± SEM) of control and retinoid-fed mice were 0.79 ± 0.03 and 0.69 ± 0.02, respectively.

Because RA inhibited the growth of LSTRA cells in vivo, we compared the ability of three other retinoids to protect mice against LSTRA lymphoma growth. The groups of BALB/c euthymic mice fed RA, cRA, 4-HPR, and RL for 2 weeks and then challenged with \(10^7\) LSTRA lymphoma cells had significantly less mortality than mice fed a control diet (Fig. 6). Mice fed 50 mg/kg of RL, RA, cRA, or 4-HPR had survival rates of 70, 50, 40, and 30%, respectively. All four retinoids had a dose-dependent effect on mortality caused by LSTRA growth. The hierarchy of retinoid-induced antilymphoma activity was RL > RA > cRA > 4-HRP. All mice that survived for 70 days post-LSTRA challenge were clinically healthy when killed 115 days after LSTRA challenge.

**RESULTS**

**LSTRA Immunization.** To evaluate the immunogenicity of LSTRA lymphoma cells, euthymic BALB/c mice were fed a normal diet and inoculated i.p. with graded doses (10^3, 10^4, 10^5) of irradiated LSTRA lymphoma cells or with PBS as a control. Two weeks after LSTRA immunization mice were challenged with 10^5 or 10^6 LSTRA cells and mortality was observed (Fig. 2). All mice survived that were immunized with 10^6 or 10^5 LSTRA cells and challenged with 10^4 or 10^3 cells, respectively. There were 40% survivors in the group of mice immunized with 10^4 lymphoma cells and challenged with 10^3 cells. The group immunized with 10^3 LSTRA and challenged with 10^4 cells had 30% survivors. When given injections of PBS and challenged with 10^5 or 10^4 LSTRA cells, all animals died within 25 days postchallenge. Similar mortality was observed in the group immunized and challenged with 10^4 lymphoma cells.

**In Vivo Effects of Retinoids on Tumor Growth.** RA had a dose-dependent effect on mortality with 50 and 10% survivors at 70 days post-LSTRA inoculation in the groups fed 50 and 12.5 mg of RA/kg of diet, respectively (Fig. 3). Mice fed 150 mg of RA/kg of diet died at a rate similar to that of controls, probably due to toxicity induced by the high RA concentration in the diet (40). All mice fed the control diet died within 42 days postinoculation.

To determine the role played by T-cells in mediating the retinoid-induced protection against LSTRA tumor growth, BALB/c athymic (nu/nu) and euthymic (nu/+ ) mice were fed either 25 mg of RA/kg of diet or a control diet for 2 weeks and challenged with 5 \times 10^7 LSTRA cells. All athymic mice fed RA or control diet died at a similar rate by 30 days after injection of tumor cells. All the euthymic mice on a control diet died within 60 days. However, the group of euthymic animals fed RA had 40% survivors at 70 days post-LSTRA challenge (Fig. 4).

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RA at 10^{-10} to 10^{-6} M had no effect on the in vitro growth of LSTRA or MSC cells during 2 to 5 days of culture as determined by DNA incorporation of [\(\text{H}\)dThd]. However, experiments to evaluate the effects of retinoids on the differentiation of MSC and LSTRA cells were not done.

**RA Effects on LSTRA Immunization.** Because retinoids have been shown to potentiate various immune functions, we evaluated the ability of RA to potentiate the immunization against...
RETINOID INHIBITION OF MURINE RETROVIRUS-INDUCED TUMOR GROWTH

Fig. 3. In vivo effect of RA on LSTRA lymphoma growth. Six mice/group were fed for 3 weeks either a control diet or a diet containing RA at 150, 50, or 12.5 mg/kg and inoculated with $10^7$ LSTRA. RA at 50 and 12.5 mg/kg of diet increased survivors in comparison to mice on control diet. RA at the high dose was toxic.

Fig. 4. Thymic dependence of RA-induced antilymphoma effect. Euthymic and athymic BALB/c mice were fed either a control diet or a diet containing 25 mg/kg RA for 2 weeks and given injections of $5 \times 10^5$ LSTRA cells. Mice were observed for deaths for 70 days. RA-induced protection against lymphoma growth was thymus dependent.

Fig. 5. In vivo effects of cRA on MSC growth. BALB/c mice were fed either a control diet or a diet containing 50 mg of cRA/kg of diet for 2 weeks and inoculated with $10^8$ MSC in the thigh. Mice fed cRA had a significant reduction in tumor burden. Tumor growth values are expressed as geometric mean increase (mm) from six animals/group. On day 11, thigh diameters (log$_e$ mean ± SEM) were significantly different ($P < 0.025$) in mice fed cRA versus control diet and were $0.69 ± 0.02$ and $0.79 ± 0.03$, respectively.

Fig. 6. In vivo comparison of antilymphoma effect of retinoids. BALB/c mice were fed for 2 weeks either a control diet or a diet containing retinoid at two different concentrations (12.5 or 50 mg/kg diet). Mice were given injections of $10^7$ LSTRA lymphoma cells after 2 weeks on retinoid diet and mortality was recorded for 70 days. High dose RL protected mice best against lymphoma growth.

Fig. 7. Effect of dietary RA on cell-mediated cytotoxicity against LSTRA. BALB/c mice (6/group) were fed either a control diet or a diet containing 50 mg of RA/kg of diet for 2 weeks and then given injections of $10^6$ irradiated LSTRA lymphoma cells. On day 4, splenocytes from each mouse were assayed for cytotoxicity against $^{51}$Cr-labeled LSTRA cells in a 4-h assay. RA caused a significant ($P < 0.05$) increase in cell-mediated cytotoxicity to LSTRA at all E:T ratios. Values are expressed as percentage of lysis ± SEM of LSTRA cells by splenocytes from three different mice.

LSTRA lymphoma cells. To determine the effect of RA on cell-mediated cytotoxicity against LSTRA, mice were fed RA or a control diet during primary immunization, and the ability of their spleen cells to lyse LSTRA cells was evaluated. Splenocytes from RA-treated mice collected 4 days post-LSTRA immunization had significantly ($P < 0.05$) increased cell-mediated cytotoxicity in comparison to immune splenocytes from animals fed a normal diet (Fig. 7). The lytic activity of splenocytes from control mice was 51.5 ± 2.0%, 63.0 ± 3.0%, and 64.0 ± 2.5% for E:T ratios of 12.5:1, 25:1, and 50:1, respectively. Spleen cells from RA-fed mice had lytic activity of 70.0 ± 2.5%, 75.0 ± 2.0%, and 78.0 ± 2.0% for E:T ratios of 12.5:1, 25:1, and 50:1, respectively.

To test whether this increased primary immunity was carried over as an anamnestic response that would protect immunized animals against challenge, we immunized animals once with irradiated LSTRA cells during a 3-week course of dietary retinoids and then inoculated them with $10^7$ living LSTRA cells.
2 weeks after immunization (1 week after resuming a normal diet). Surprisingly, dietary RA administered during immunization caused a dose-dependent decrease in the number of survivors after challenge with 10^5 LSTRA cells (Fig. 8). The groups of mice immunized with 10^5 LSTRA cells and challenged with 10^3 cells had different survival rates, depending on the concentration of RA in the diet with 100, 80, or 50% survivors in groups fed 0, 12.5, or 50.0 mg of RA/kg of diet, respectively.

Animals immunized with 10^5 LSTRA while fed a diet containing 0, 12.5, or 50.0 mg of RA/kg of diet and challenged with 10^3 LSTRA had survival rates of 50, 33, and 17%, respectively. Mice immunized with 10^5 LSTRA cells while fed 0, 12.5, or 50.0 mg of RA/kg of diet and challenged with 10^2 LSTRA had survival rates of 17, 17, and 0%, respectively.

DISCUSSION

In this report we have shown that dietary retinoids can protect previously unimmunized animals from challenge with two related murine retrovirus-induced tumors by a thymus-dependent mechanism. We confirmed previous studies showing that both LSTRA lymphoma cells and MSC sarcoma cells are immunogenic in syngeneic hosts (24, 27, 32, 34, 35). MSC-induced tumors regressed and mice were protected against LSTRA growth by preimmunization with lymphoma cells. The level of protection against lymphoma growth was dependent on the difference between the number of cells used for immunization versus the number used in the challenge dose. For example, 100% of mice survived that were immunized with 10^6 irradiated LSTRA cells and challenged with 10^6 cells. Whereas no animals survived an immunization dose of 10^4 cells and subsequent challenge with 10^4 cells. LSTRA lymphoma cells are known to express M-MuLV-specific cell surface antigens (32) that cause the tumor to be immunogenic in syngeneic hosts. MSC cells also express M-MuLV-specified antigens that evoke strong cellular (24) and humoral (27) immune responses that result in regression of the tumor in appropriate hosts. Because these retroviral-induced tumors are immunogenic, they provide a suitable system in which to evaluate the chemoprotective effects of retinoids against immunogenic tumors in syngeneic hosts.

Retinoid-induced inhibition of transplantable tumor growth in syngeneic hosts has been observed by Eccles et al. (7) and Patek et al. (10) most often with tumors capable of inducing an immune response. Retinoids have inhibited immunogenic transplantable tumors induced by chemical carcinogens (41) or viruses (42, 43). Giese et al. (42) and Seifter et al. (43) have reported that dietary vitamin A palmitate and topically applied RA, respectively, have been shown to cause a significant decrease in the incidence and size of M-MuSV-induced tumors. Our results confirm the ability of another retinoid, cRA, to inhibit the growth of an M-MuSV-induced tumor.

Our evaluation of the effect of RA on the growth of another transplantable retroviral-induced tumor that does not spontaneously regress, LSTRA, showed that an optimal dietary concentration of RA could significantly decrease LSTRA growth with total tumor resistance in a large proportion of the treated animals. The specific mechanisms by which retinoids inhibit tumor growth in vivo are unknown, but may involve either direct effects of retinoids on tumor cells and/or indirect mechanisms such as increasing antitumor immune responses (4, 9). Our data suggest that RA inhibits LSTRA growth through cell-mediated immune responses, since RA had no effect on LSTRA growth in athymic (nu/nu) mice, but euthymic mice fed RA were protected against lymphoma growth. Also, normal mice fed RA had increased cell-mediated cytotoxicity against LSTRA cells when compared to mice fed a control diet. The finding that RA did not inhibit the growth of LSTRA in vitro also implies that the antilymphoma effect is at the level of host resistance and probably not due to a direct antiproliferative effect on the tumor cells. However, these studies do not exclude possible effects of RA on the differentiation of these tumor cells. Other reports have shown that the inhibitory effects of retinoids on growth of transplantable tumors were abolished in animals immunocompromised by whole body irradiation, cyclosporin A, antilymphocyte serum, or neonatal thymectomy (9, 10, 44).

Eccles et al. (45) and others (24, 46) have reported that mice gavaged with retinoids have an increase in the number of mononuclear cells within their tumors compared to tumors removed from control mice. This suggests that the antitumor effects of retinoids may be due to an enhancement of local cell-mediated immune responses. In MSC-induced sarcomas, T-lymphocytes and macrophages are the predominant tumor-infiltrating cell type in regressing tumors (47). Since retinoids in vitro have been shown to potentiate the proliferative (39) and cytotoxic activity of T-cells (16) and enhance macrophage phagocytosis (48) and antitumor activity (15, 49), these cellular mechanisms may also be involved in the in vivo antitumor effect of retinoids. It has been reported that vitamin A in vivo caused the rejection of a syngeneic fibrosarcoma by enhancing the cytotoxic activity of Thy-1-positive, Lyt-2-positive, Lyt-1-negative lymphocytes (50). Similarly, Forni et al. (18) have recently shown that the potentiation of various cellular immune functions by dietary retinyl palmitate paralleled the growth inhibition of three transplantable tumors. Thus, retinoids appear to inhibit the growth of immunogenic tumors through potentiation of cellular immune responses.

Comparison of the ability of different retinoids to prolong the survival of mice inoculated with LSTRA lymphoma cells showed a hierarchy of RL > RA > cRA > 4-HPR. RL is a newly synthesized retinoylamino acid (38) that has recently been shown to have less toxicity and more immunopotententiating activity than RA (51). The results in the present study indicate that RL is the most active compound tested in terms of providing resistance to lethal lymphoma development following inoculation of LSTRA cells.

Because retinoids have been shown to have an adjuvant effect on various immune responses (11), we evaluated the effects of
dietary RA on the immunization of mice with irradiated LSTRA cells. Animals immunized i.p. with 10⁶ irradiated LSTRA cells following 2 weeks of dietary RA developed significantly more active lytic effector cells in their spleens against the immunizing target cells than comparably immunized animals that had been maintained on a control diet. This capacity to develop increased cell-mediated cytotoxicity in response to primary immunization may be one mechanism responsible for the in vivo resistance to lethal lymphoma development by animals given dietary retinoids.

We also conducted experiments to determine if preimmunization of animals during a regimen of dietary retinoids would have an effect on their susceptibility to challenge after supplemental retinoids were discontinued. Animals preimmunized while being fed a control diet were more resistant to subsequent challenge than were the RA-fed groups. Thus, these experiments imply that RA causes a dose-dependent decrease in immunological memory to LSTRA cells since mortality was higher in those immunized animals fed RA versus animals fed a control diet. This observation appeared somewhat paradoxical. However, a similar observation of retinoid-induced inhibition of immunological memory was made by Medawar and Hunt (52) who used syngeneic male to female skin transplants. In their study, dietary retinyl acetate increased the rate of primary rejection. In contrast, mice fed retinoid during the first transplant and then regrafted with an identical skin graft had no detectable secondary response, i.e., accelerated graft rejection observed with regrafted control animals was not observed among the animals that had rejected their primary grafts during retinoid treatment. The mechanisms by which retinoids seem to inhibit immunological memory are unknown. One possibility is that the presence of RA during LSTRA immunization may induce potential memory cells to differentiate to terminal effector cells. This would effectively deplete the animal of cells capable of responding to a secondary exposure to the immunizing antigens. This possible mechanism is consistent with the reported ability of retinoids to drive normal and neoplastic cells toward a more differentiated state (16, 53, 54). A number of questions are raised by these observations, including the immunological specificity and subpopulations of lymphoid cells involved in this memory loss. Retinoids may provide a valuable tool for the study of the mechanisms of immunological memory.

We can arrive at several important conclusions from this series of experiments. Dietary retinoids can provide resistance to lethal tumor development following inoculation of syngeneic retrovirus-induced tumor cells in unimmunized animals. This resistance is thymus dependent, since athymic (nu/nu) mice fed RA were not protected against LSTRA growth as were euthymic mice. Furthermore, animals that are given a primary immunization during dietary retinoid treatment develop increased cell-mediated cytotoxicity. However, this potentiated primary response is apparently not long lasting. Indeed, immunization in the presence of supplemental retinoids seems to inhibit the development of immunological memory. Finally, dietary retinoylucelate appears to provide the best protection against syngeneic Moloney lymphoma challenge among the four retinoids we have tested in this study. This compound has been shown by us to be less toxic than RA in vivo and has immunopotentiating properties equal to or greater than RA in vitro (51).

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