Enhancement and Suppression of Murine Sarcoma Virus Induced Tumors by Polyriboinosinic·Polyribocytidylic Acid[1] (36126)

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(Introduced by S. Baron)

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The Moloney strain of murine sarcoma virus (MSV) rapidly induces tumors at the site of virus inoculation in mice of all ages (1). In the majority of immunologically competent mice spontaneous tumor regression occurs (1, 2). At appropriate dilutions, MSV induces discrete focal lesions in mouse tissue culture cells.

The synthetic polyribonucleotide polyriboinosinic·polyribocytidylic acid (rI·rC), a potent interferon inducer, can protect animals from virus-induced, chemically-induced, and transplanted syngenic tumors (3–5). Continuous treatment with rI·rC can prevent MSV tumor induction in mice and focus formation in mouse tissue culture cells (6, 7). However, recent studies indicate that rI·rC treatment is not always effective in the treatment of virus-induced and chemically-induced murine tumors (8–12). The antitumor effect of rI·rC is dependent on the age and strain of the mice used (11, 12), and is not correlated with rI·rC induced circulating interferon levels (12). We define the conditions required for rI·rC mediated suppression and enhancement of MSV tumor induction.

Methods and Results. Mice. Weanling BALB/c and AL/N mice were obtained from the National Cancer Chemotherapy Service, National Cancer Institute, Bethesda, MD. Additional AL/N mice were obtained from the Rabbit and Rodent Section, Division of Research Services, National Institutes of Health, Bethesda, MD.

Virus. MSV concentrate was prepared from virus-induced tumors in BALB/cCR mice by differential centrifugation (13) and was a 1 g/ml equivalent. Aliquots of a single pool of virus were used throughout and were stored at —70°. Unless stated otherwise, all virus inoculations consisted of $10^{1.5}$ tumor-inducing doses, as titered in newborn BALB/c mice.

rI·rC. rI·rC was purchased from P-L Biochemicals, Milwaukee, Wisconsin, as the lyophilized physiological salt, and was reconstituted with pyrogen-free distilled water to a concentration of 1 mg/ml. Hypochromicity was 38% at 248 mμ at pH 7.0.

Animal inoculations. Virus dilutions were prepared in phosphate buffered saline (PBS), pH 7.0, immediately prior to inoculation. Virus was inoculated into the left thigh by the subcutaneous–intramuscular route. The rI·rC was inoculated by the same route into either the right or the left thigh. Control mice were inoculated with the equivalent volume of PBS. Mice were examined 4, 5, 7, 9, 12, 15, 20, 25, and 30 days after virus inoculation and tumor size was carefully graded 0 to 4+, using the criteria of Blumenschein and Moloney (14). Each experimental and control group consisted of 10 mice.

Local vs. systemic injection of rI·rC. The effects of systemic injection of rI·rC on MSV tumor induction in AL/N and BALB/c mice are shown in Fig. 1. Mice received either a single injection or rI·rC 24 hr prior to virus inoculation, or injections every second day commencing 24 hr prior to virus inoculation. Pretreatment with a single dose of rI·rC enhanced tumor induction ($p<.01$) in AL/N mice, but had no significant effect in BALB/c mice ($p>.05$). The enhancement in AL/N mice (Fig. 1a) was manifested by increased tumor size, higher incidence of tumors (10/10 in the rI·rC group vs. 6/10 in

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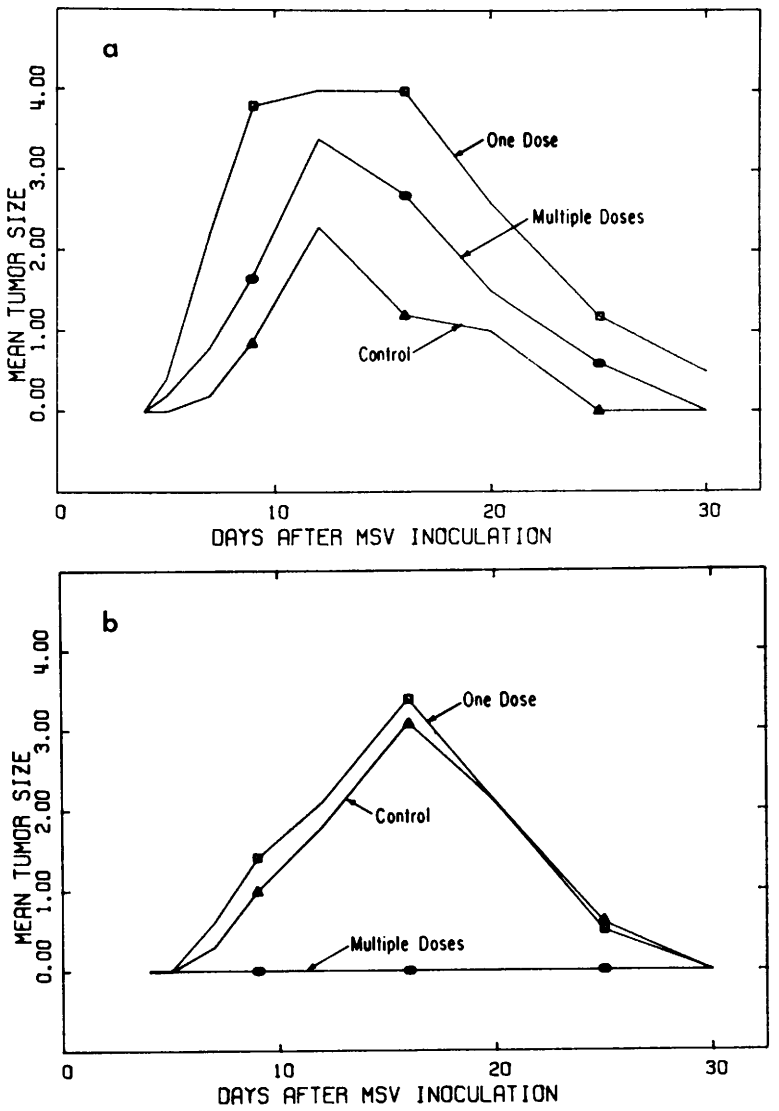

FIG. 1. Effects of systemic injection of rI·rC on MSV tumor induction in AL/N (a) and BALB/c (b) mice. Mice received either a single dose of 200 μg of rI·rC 24 hr prior to virus injection, or multiple doses, given every second day starting 24 hr prior to virus injection. The rI·rC was injected into the right thigh and the virus into the left thigh.

the control group), shorter latent time, to tumor appearance (5.8 days in the rI·rC group vs. 8.6 days in the controls) and longer time until complete tumor regression (27.6 days in the rI·rC group vs. 22.4 days in the controls). Multiple injections of rI·rC enhanced tumor induction in AL/N mice less than a single injection. However, in BALB/c mice, multiple injections completely suppressed tumor induction (all 10 control mice developed tumors). The effects of rI·rC (*i.e.*, enhancement of MSV tumor induction in AL/N mice and tumor suppression in BALB/c mice) were identical in male and female mice.

Multiple injections of rI·rC into the virus inoculation site had an opposite effect in AL/N mice than systemic injections. While pretreatment with a single injection of rI·rC 24 hr prior to virus inoculation into the same

site enhanced MSV tumor induction, multiple injections of rI·rC into the virus inoculation site suppressed tumor induction (only 2/10 of the rI·rC injected mice developed tumors after a long latent period, while 9/10 control mice developed tumors).

Single and multiple doses of rI·rC injected into the virus inoculation site in BALB/c mice had the same effects, respectively, as did systemic administration.

Inoculation of rI·rC at different times. The effects of inoculating rI·rC at different times in relation to the time of virus inoculation are presented in Fig. 2. In AL/N mice (Fig. 2a) a single dose of rI·rC administered systemically at the time of virus inoculation or

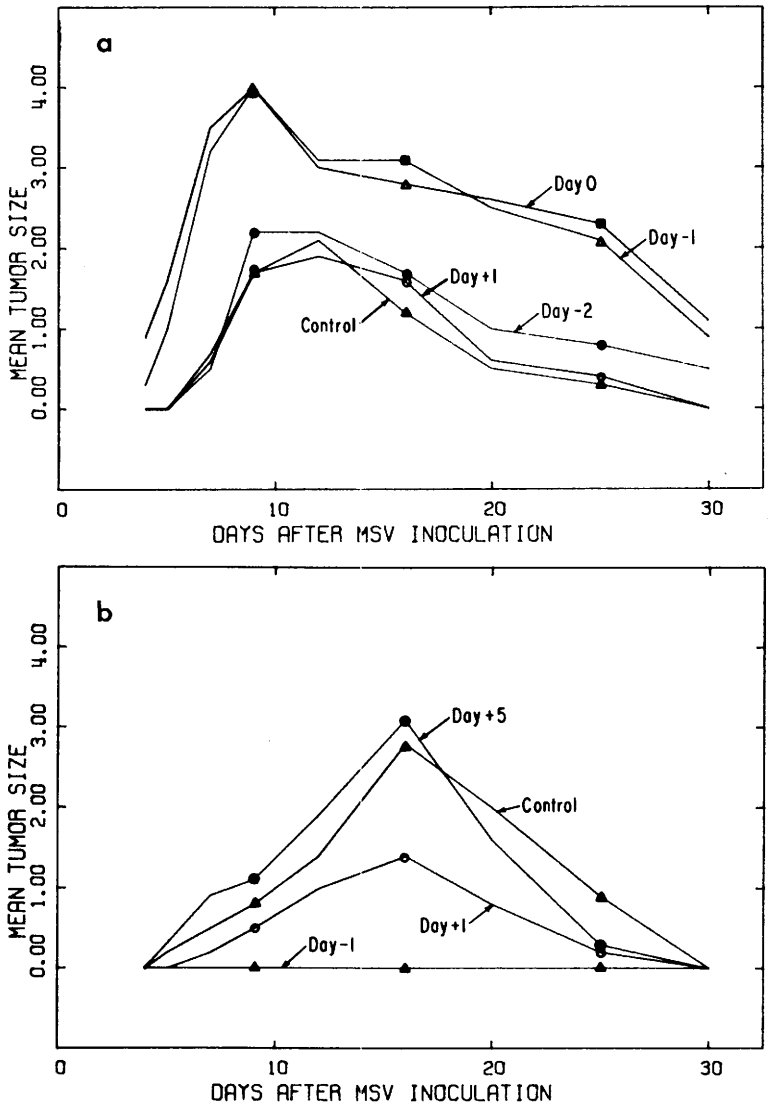

FIG. 2. Effects of time of injection of rI·rC on MSV tumor induction in AL/N (a) and BALB/c (b) mice. Each injection of rI·rC, administered systemically, consisted of 200 μg. AL/N mice received a single injection of rI·rC 1 or 2 days prior to MSV injection, simultaneously with the virus, or 1 day after the virus. BALB/c mice received injections of rI·rC every second day, commencing 1 day prior to virus injection, 1 day after virus injection, or 5 days after virus injection.

24 hr prior to virus inoculation enhanced MSV tumor induction to the same degree. Inoculation of a single dose of rI·rC into AL/N mice 48 hr before or 24 hr after virus inoculation had no significant effect. In BALB/c mice, rI·rC was injected systemically, every second day (Fig. 2b). Commencement of rI·rC injections 24 hr prior to virus inoculation completely suppressed tumor induction. Starting rI·rC injections 24 hr after virus administration only partially suppressed tumor induction. Commencement of rI·rC treatment 5 days after virus inoculation, when the first tumors were palpable, had no effect.

Relationship between dose of rI·rC and effect. Experiments were performed to determine the minimal effective dose of rI·rC. In AL/N mice pretreatment with a single dose of 200 μg enhanced MSV tumor induction to a greater extent than pretreatment with a single dose of 20 μg. Pretreatment with 2 μg had no effect on tumor induction. BALB/c mice received rI·rC injections systemically every second day commencing 24 hr prior to virus inoculation. Injections of 200 μg of rI·rC completely suppressed tumor induction, and injections of 100 μg partially suppressed tumor induction, but injections of 50 μg and 20 μg were ineffective.

Effect of virus dose. To determine the effect of virus dose on rI·rC mediated enhancement and suppression of MSV tumor induction, AL/N and BALB/c mice were inoculated with $10^{1.5}$, $10^{2.5}$, and $10^{3.5}$ tumor inducing doses of MSV. Injection of $10^{1.5}$ tumor inducing doses, as titered in newborn BALB/c mice, represents the minimal amount of virus required for an 80–100% tumor incidence in weanling mice. AL/N mice were pretreated with a single dose of 200 μg of rI·rC 24 hr prior to virus inoculation. Enhancement occurred in all the rI·rC treated groups, but was minimal in the mice receiving $10^{3.5}$ tumor inducing doses. BALB/c mice received 200 μg of rI·rC injected systemically every second day starting 24 hr prior to virus inoculation. Tumor suppression by rI·rC occurred in mice receiving $10^{1.5}$ tumor inducing units, but not in mice receiving higher virus doses.

Tumor occurrence after continuous rI·rC treatment. As described above, continuous treatment with rI·rC for 30 days suppressed MSV tumor induction in BALB/c mice. BALB/c mice were injected with rI·rC every second day for 28 or 42 days, and observed for 63 days. The results, presented in Table I, indicate that tumors developed shortly after cessation of prolonged rI·rC treatment. Tumors developed only after rI·rC treatment had been stopped. The latent times for tumor development after cessation of rI·rC were similar to the latent time in the controls.

TABLE I. Tumor Occurrence in BALB/c Mice After Continuous rI·rC Treatment.[a]

Duration of rI·rC injections (days)	No. of tumors/ no. of mice inoculated	Mean latent time to palpable tumors (days)
—	10/10	6.7
28	10/10	35.1
42	8/10	50.4

[a] Weanling BALB/c mice received systemic injections of 200 μg of rI·rC every second day commencing 24 hr prior to MSV inoculation. The observation period was 63 days.

Effect of immunization with double-stranded RNA. To determine whether the enhancement of tumor induction by rI·rC was a specific action of the polyribonucleotide, 20 AL/N female mice were immunized by an intraperitoneal injection of 100 μg of polyriboadenylic·polyribouridylic acid (P-L Biochemicals, Milwaukee, WI) emulsified in an equal volume of Freund's complete adjuvant (inoculum volume = 0.2 ml/mouse). Twenty control mice were injected with the same volume of Freund's complete adjuvant. Seven days later the mice were bled orbitally and the serum levels of antibody to double-stranded RNA determined by an ammonium-sulfate precipitation assay using ^{14}C-labelled rI·rC as described previously (15). The mean values, expressed as amount of rI·rC bound by serum were: immune mice = 120 μg/ml; control mice = 6.1 μg/ml. The levels of antibody present in the immunized mice can neutralize the interferon inducing action of rI·rC (16). On the seventh day after immu-

nization, 10 of the immunized and 10 of the control mice were injected with 200 μg of rI·rC. The remaining mice received PBS. Twenty-four hours later all the mice received $10^{1.5}$ tumor-inducing doses of MSV. As shown in Fig. 3, rI·rC pretreatment produced (Table II). In addition, rI·rC treated mice had enlarged spleens due to hyperplasia of the red pulp and lymphoid follicles, and their thymuses were reduced in size with partial depletion of cortical lymphocytes. The hearts of all 10 mice treated with rI·rC had exten-

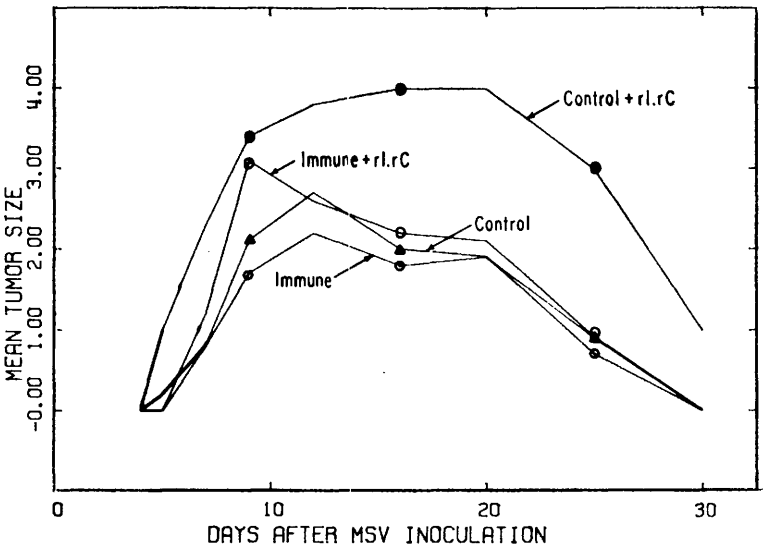

FIG. 3. Effect of prior immunization with double-stranded RNA on rI·rC enhancement of MSV tumor induction in AL/N female mice. Antibodies to double-stranded RNA were induced by immunization with intraperitoneal injection of 100 μg of polyriboadenylic·polyribouridylic acid emulsified in Freund's complete adjuvant. Seven days later immune and adjuvant-injected control mice were injected in the right thigh with 200 μg of rI·rC or PBS. The next day all mice received an injection of MSV in the left thigh.

significantly less tumor enhancement in the immune group than in the control group.

Toxicity of rI·rC. The toxic effects of the tumor enhancing and suppressing rI·rC regimens were investigated in weanling AL/N and BALB/c mice, respectively. AL/N mice received a single injection of 200 μg of rI·rC, while BALB/c mice received injections every second day. Control mice received PBS. The mice were weighed weekly. After 14 or 30 days, the AL/N and BALB/c mice, respectively, were sacrificed, their spleens and thymuses weighed, and the major organs examined grossly and microscopically. A single injection of rI·rC had no significant effect on the body, spleen, and thymus weights of AL/N mice, and no gross or microscopical lesions were noted in their major organs. BALB/c mice given multiple injections of rI·rC weighed 12–15% less than control mice sive calcification limited to the antero-median surface of the right ventricle and the overlying pericardium. The calcification caused a giant-cell granulomatous reaction. One mouse also had calcification in the red pulp of the spleen. Calcification was not present in any of the control mice.

Discussion. De Clercq and Merrigan demonstrated the influence of the age of mice on the effect of rI·rC on MSV tumor induction (11). Our studies indicate that the relationship between rI·rC and MSV tumor induction is highly complex and depends on multiple factors including the strain of mouse, dose and time of administration of rI·rC, number of injections of rI·rC, site of injection of rI·rC, and dose of MSV. In fact, rI·rC had opposite effects in two different strains of mice, producing suppression of tumor growth in BALB/c mice and enhance-

TABLE II. Toxicity of rI • rC in Weanling Mice.

Mouse strain	% Weight change						
	Body weight					Spleen weight	Thymus weight
	Day 0	Day 7	Day 14	Day 21	Day 28		
BALB/c[a]	+0.7[c] ($p > .05$)	−12.7 ($p < .001$)	−14.2 ($p < .001$)	−15.2 ($p < .001$)	−12.3 ($p < .001$)	+64.8 ($p < .001$)	−21.7 ($p < .005$)
AL/N[b]	−0.2 ($p > .05$)	−6.7 ($p > .05$)	−4.1 ($p > .05$)			+3.4 ($p > .05$)	−5.4 ($p > .05$)

[a] BALB/c mice were injected with 200 μg of rI • rC every second day and sacrificed after 30 days.
[b] AL/N mice received a single injection of 200 μg of rI • rC and were sacrificed after 14 days.
[c] The figures represent the mean % weight changes of the rI • rC injected mice compared to PBS injected controls. Control and rI • rC injected groups consisted of 10 mice each. The p values were calculated by the Student-Fisher T test.

ment in AL/N mice. Suppression of tumor enhancement in BALB/c mice required multiple injections of rI·rC commencing one day prior to virus inoculation. The effective therapeutic regimen was highly toxic, resulting in depression of body and thymus weights, and an increase in spleen weight. These toxic effects have been described before (17). Dystrophic calcification of the right ventricle is a common finding in aged mice of several strains (18). NIH Swiss mice receiving multiple injections of rI·rC developed calcification along the anterior hepatic border (A. F. Gazdar, A. J. Weinstein, and H. Sims, unpublished observations). As hepatic calcification is not a known aging process, calcification induced by rI·rC probably represents a species-specific toxic effect and not an acceleration of a normal aging process. As suppression of MSV tumor induction occurred only after repeated injections of toxic doses of rI·rC, and only at terminal virus dilutions, the tumor suppressive action of rI·rC may be mediated by its toxic effects on the host. Further, rI·rC may have a direct chemotherapeutic effect, as Levy *et al.*, reported that rI·rC inhibits macromolecular synthesis in tumor cells (19). The appearance of virus-induced leukemias (10) and MSV-induced sarcomas after cessation of prolonged rI·rC administration suggests that the viruses are not completely suppressed by this treatment. The lack of correlation between induced interferon levels and the antitumor effect of rI·rC has been reported (12). The interferon levels induced by rI·rC in BALB/c and AL/N mice are similar (authors' unpublished data).

In contrast to suppression, tumor enhancement occurred in AL/N mice from single, subtoxic doses of rI·rC injected simultaneously with or 24 hr prior to MSV injection. Tumor enhancement is a specific action of rI·rC, as demonstrated by its reversal in mice previously immunized with double-stranded RNA. Enhancement appeared to be a more potent action of rI·rC than suppression, because it was produced by single subtoxic doses, and occurred at all virus dilutions. In contrast, tumor suppression required multiple injections of rI·rC, and occurred only at terminal virus dilutions. Both tumor enhancement and suppression by rI·rC have been attributed to its adjuvant-like action on the immune system (11, 20). However, the very short latent period for MSV tumor induction suggests that other mechanisms are responsible for rI·rC mediated enhancement. Conceivably, pretreatment with rI·rC may have some action on virus–target cell interaction or selectively aid tumor cell growth. Tumor enhancement and suppression by rI·rC are due to separate mechanisms, as both actions could be demonstrated in AL/N mice.

The present studies indicate that the effect of rI·rC on virus-induced tumors is not a simple one. Further study is required for a better understanding of both the suppression and enhancement of tumor formation by rI·rC.

Summary. This paper reports the effects of polyriboinosinic·polyribocytidylic acid (rI·rC) on the Moloney strain of murine sarcoma virus (MSV) in BALB/c and AL/N mice. Complete suppression of tumor induction in BALB/c mice was produced by a highly toxic regimen, using terminal virus dilutions. Furthermore, tumors occurred in BALB/c mice after the cessation of multiple injections of rI·rC. These findings suggest that the tumor suppressive action of rI·rC is more likely mediated by its toxic or chemotherapeutic effects than by a direct antiviral action. Pretreatment with a single subtoxic dose of rI·rC, or simultaneous administration of rI·rC and MSV, resulted in enhancement of tumor induction in AL/N mice at all virus dilutions tested. The enhancement was a specific action of rI·rC as it could be reversed by prior immunization with double-stranded RNA. These studies suggest that the relationship between host, drug, and virus is dependent on multiple factors and is more complex than previously thought.

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