Synthetic RNA and DNA Polynucleotides: *in Vivo* and *in Vitro* Enhancement of Oncogenesis by a Murine Sarcoma Virus (36633)

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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 and, at appropriate dilutions, induces discrete foci of transformed cells in mouse tissue culture cells (1, 2). High doses of, or continuous treatment with interferon and interferon inducers can suppress MSV tumor induction and focus formation (3–6). However, recent studies demonstrate the highly complex nature of the role of interferon inducers in viral oncogenesis (7–11). Depending on the conditions, several interferon inducers are capable of either enhancing or suppressing MSV tumor induction (11). We herein report the *in vivo* and *in vitro* enhancement of oncogenesis by MSV by several synthetic polyribo- and polydeoxyribonucleotides.

Materials and Methods. Mice. Weanling 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 by differential centrifugation (12) and was a 1 g of tumor/1 ml of virus equivalent. A single pool was used for the *in vivo* studies. The virus stock used for focus assays was prepared by superinfecting S+L− cells (13) with Moloney leukemia virus.

Animal inoculation. MSV was injected by the subcutaneous–intramuscular route. A dose that induces small tumors in approximately 60% of weanling mice was used (approximately 10^3 focus-forming units). Mice were examined 4, 5, 7, 9, 12, 15, 20, 25, and 30 days after virus inoculation and tumor size was carefully graded 0–4+, using the criteria of Blumenschein and Moloney (14). Each group consisted of ten mice.

Nucleotides. The synthetic nucleotides rI·rC, rA·rU, rA·rU·rU, rI, rC, adenosine monophosphate (AMP), adenosine triphosphate (ATP), adenosine cyclic monophosphate (cAMP), and dibutyryl adenosine cyclic monophosphate (dbcAMP) were purchased from P–L Biochemicals, Milwaukee, WI. The synthetic nucleotides d(A–T), dG·dC, dT, and rA·dT were obtained from Miles Laboratories, Kankakee, Ill. Calf thymus DNA was purchased from Worthington Biochemicals, Freehold, NJ. Mice were injected intraperitoneally with 200 μg of nucleotide 24 hr prior to virus inoculation. Some nucleotides were supplied in OD units, and the actual amounts used may vary slightly from the stated amount (±10%).

Focus assay. Focus formation by MSV in 3T3FL cells, a subline of the original 3T3 Swiss mouse cell line (15), was determined by a modification of the method previously described (13). 3T3FL cells, obtained from Dr. R. Bassin, National Cancer Institute, were grown in Eagles minimum essential medium, Earle's base, (EMEM), (GIBCO, Grand Island, NY) supplemented with 10% heat inactivated fetal bovine serum and antibiotics. They were plated onto 60 × 15 mm plastic dishes (Falcon Plastics, Oxnard, CA) at a concentration of 1×10^5 cells per dish. After 24 hr the supernatant fluids were removed and the dishes treated with 1 ml of nucleotide dissolved in EMEM with serum for 2 hr after which 3 ml of complete medium were added. The nucleotide solution was removed after 24 hr, the cells rinsed once with phosphate buffered saline, and infected with MSV (0.2 ml volume). After 1 hr, 4 ml of

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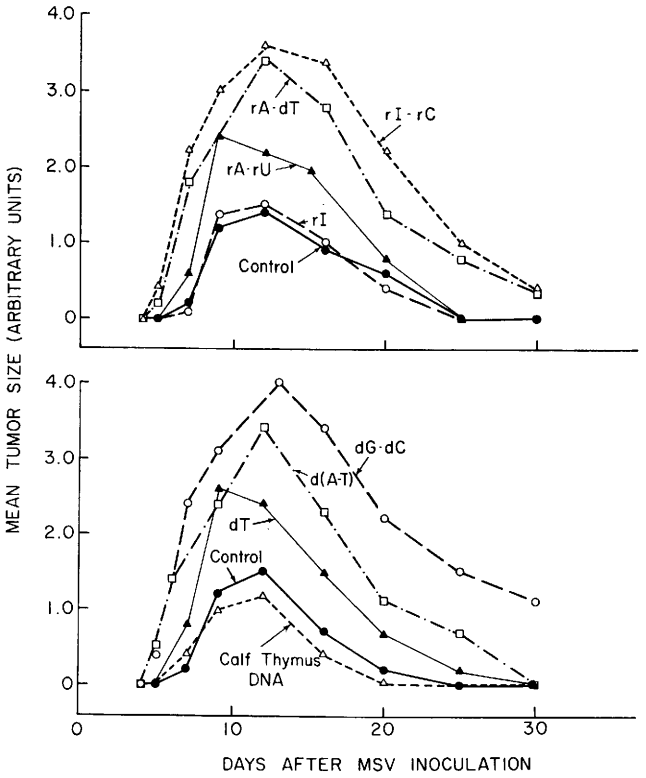

FIG. 1. Effects of polynucleotide pretreatment on MSV tumor induction. Mice were injected intraperitoneally with 200 μg of polynucleotide 24 hr prior to MSV injection into the left thigh.

complete medium were added. The plates were kept at 37° in a humidified 5% CO_2 atmosphere. Foci were counted 5 days after infection. DEAE–dextran pretreatment of the cells was omitted.

Results. Enhnacement of tumor growth. Figure 1 depicts the effects of nucleotide pretreatment on MSV tumor induction. The polynucleotides rI·rC, rA·dT, rA·rU, d(A–T), dG·dC, and dT enhanced tumor induction as demonstrated by shorter latent period, larger mean tumor size, higher tumor incidence, and longer time for complete regression. Pretreatment with calf thymus DNA, rI, rC, dA, rA·rU·rU, AMP, ATP, cAMP and dbcAMP had no effect on MSV tumor induction.

Enhancement of focus formation. As shown in the representative results presented in Fig. 2, rI·rC and d(A–T) pretreatment of 3T3FL cells resulted in a 10–30 fold increase in MSV focus formation. Pretreatment with 5 μg of rI·rC enhanced forcus formation to a lesser extent than pretreatment with 0.5 and 0.05 μg (Fig. 2A), while pretreatment with 50 μg of rI·rC had no effect. The foci in the control plates were fairly uniform in size, but focus size after polynucleotide pretreatment varied considerably, some foci being very large. Pretreatment with 50 μg of rI·rC had a toxic effect on the cell sheet, as manifested by increased granularity of the cells and the appearance of cellular debris in the supernatant fluids.

Discussion. Previous studies demonstrated that pretreatment with several interferon inducers of widely differing chemical composition and origin enhanced oncogenesis by RNA leukemia and sarcoma viruses (11). Tumor enhancement by rI·rC was not related to its adjuvant-like effects on humoral immunity, and was independent of the induced circulating interferon levels (6, 10). Our present studies, demonstrating enhancement of the *in vitro* effects of MSV by polynucleo-

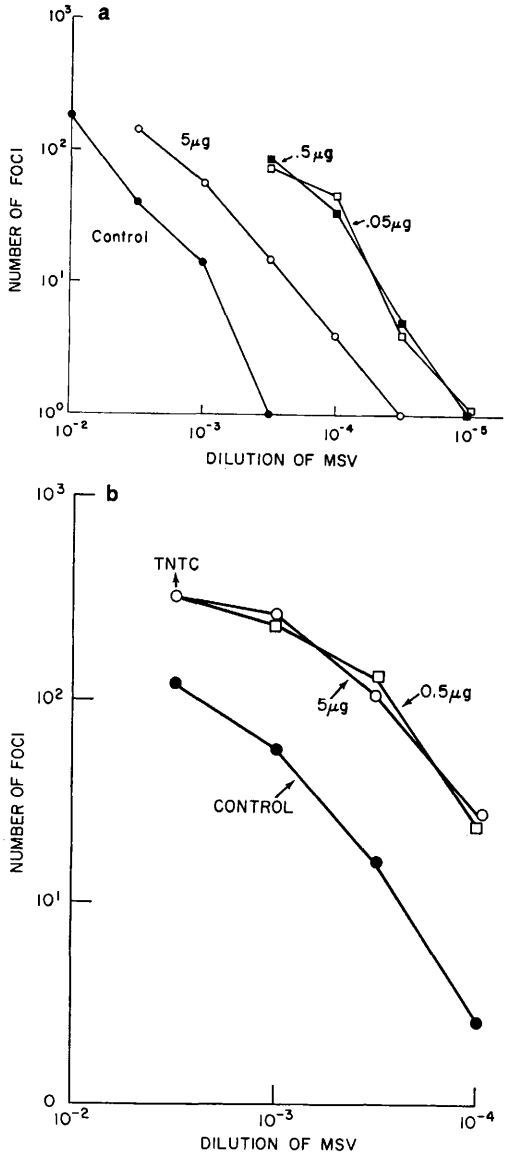

FIG. 2. Effects of polynucleotide pretreatment on MSV focus formation. 3T3FL cells were pretreated with rI·rC (Fig. 2A) or d(A–T) (Fig. 2b) 24 hr prior to injection with MSV. Foci were enumerated 5 days after infection.

tide pretreatment, provide further evidence that tumor enhancement cannot be mediated solely via *in vivo* operative mechanisms such as the immune system.

The mechanisms by which polynucleotides may enhance MSV focus formation include increased viral adsorption or penetration, increased efficiency of viral transformation, increased rate of viral replication and spread, and increased rate of growth of transformed cells.

Polycations enhance the sensitivity of tissue culture assays for certain viruses including several strains of RNA leukemia and sarcoma viruses (16). Pretreatment with the polycation DEAE–dextran enhances tumor induction as well as focus formation by MSV (17). Viral enhancement by polycations can be explained on the basis of viral and cellular surface charges (16). The net cell surface charge is negative and polycations, by binding to the cell surface, may enhance the adsorption and penetration of negatively charged virions, while polyanions inhibit these events (16). Polynucleotides are polyanions, and enhancement of MSV oncogenesis by them cannot be explained on the basis of surface charge phenomena, or by neutralization of polyanions. DEAE–dextran enhances MSV tumors if administered simultaneously with or 1 hr prior to the virus (17), but is ineffective if administered 24 hr prior to MSV (unpublished data of A.F.G.). However, polynucleotides enhance MSV tumors if administered 24 hr prior to, or simultaneously with the virus (10).

Rhim *et al.* have reported suppression of MSV focus formation in mouse embryo cells after 24 hr pretreatment of the cells with 25–100 μg of rI·rC (4). While our results appear contradictory to their findings, we found that pretreatment with 50 μg of rI·rC was toxic to 3T3FL cells. Furthermore, enhancement of focus formation by rI·rC was greater after pretreatment with 0.5 and 0.05 μg than with 5 μg. This may reflect subtle toxic effects induced by pretreatment with 5 μg.

Both MSV replication and focus formation are dependent on host cell multiplication, and any substance that reduces the rate of cellular division will reduce MSV focus formation. Suppression of MSV oncogenesis by high doses of or continuous treatment with rI·rC may be mediated by a direct toxic or chemotherapeutic effect of the drug on the host and/or the tumor cell (10).

The double-stranded polyribonucleotides

rI·rC and rA·rU are potent interferon inducers, but double and single stranded deoxyribonucleotides and single stranded ribonucleotides and deoxyribo–ribonucleotide hybrids are not established interferon inducers. However, d(A–T), dG·dC, and rA·dT did induce relatively small and variable levels of circulating interferon in mice while rI, rC, rA·rU·rU, ATP, cAMP, dbcAMP, and calf thymus DNA did not (unpublished observations of A.F.G. and S. Baron). There appears to be complete correlation between the interferon inducing and MSV enhancing properties of polynucleotides. While a potential link between the interferon inducing and MSV enhancing properties of polynucleotides exists, there is no direct correlation between the potency of these two properties. Thus the relatively poor interferon inducers d(A–T), rA·T and dG·dC enhanced MSV tumors as effectively as the potent interferon inducers rI·rC and rA·rU. This concept is supported by the previous findings of a lack of correlation between rI·rC induced circulating interferon responses and MSV tumor enhancement (11) and suppression (6).

The mechanism of MSV enhancement by polynucleotides remains unknown, but the demonstration of this effect *in vitro* may eventually shed further light on the complex interrelationships between polynucleotide, host cell, tumor virus and interferon induction.

Summary. Pretreatment with the synthetic polynucleotides rI·rC, rA·rU, d(A–T), dG·dC, rA·dT and dT enhanced tumor induction in mice by the Moloney strain of murine sarcoma virus (MSV), but pretreatment with rI, rC, rA·rU·rU and calf thymus DNA had no effect. In addition, pretreatment with rI·rC and d(A–T) enhanced focus formation by MSV 10–30 fold in mouse tissue culture cells. A correlation may exist between the MSV enhancing and the antiviral properties of polynucleotides.

Note added in press: Preliminary characterization of the antiviral substance induced by d(A-T) indicates that its properties are not similar to those of mouse interferon (A.F. G. and S. Baron, unpublished observations).

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