Antitumor Effects of Polynucleotides and Theophylline

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SUMMARY

Polyadenylic-polyuridylic acid and polyinosinic-poly-cytidylic acid, as well as theophylline, retard the rate of intradermal growth of Rauscher leukemia virus-induced ascites tumor cells in syngeneic BALB/c mice. The combination of polyadenylic-polyuridylic acid and theophylline tends to affect the growth more than either agent alone, the results being dependent on dosages, timing, and the tumor cell line used. A substantial part of the antitumor activity of these agents, which are also known to modify immune responses, appears to be due to a direct effect on the tumor cells, since the effects of polyadenylic-polyuridylic acid and theophylline were also observed in irradiated mice and usually also after direct treatment of the tumor cells in vitro prior to implantation. In view of data from other cell systems, which show that synthetic polynucleotides stimulate the enzyme responsible for the formation of cyclic adenosine 3',5'-monophosphate and also in view of the fact that theophylline inhibits the enzymes that degrade cyclic adenosine 3',5'-monophosphate, the inhibitory effects noted here are believed to involve alterations in the cyclic adenosine 3',5'-monophosphate system of the tumor cells.

INTRODUCTION

Double-stranded synthetic polynucleotides, particularly poly(I, C), show antitumor effects in a number of tumor systems (1, 12, 14, 17, 23–25, 29, 33). We have previously reported (3, 6, 7) that poly(A, U) has similar effects, at least against MC murine tumor cell line, and we have emphasized that poly(A, U), in contrast to poly(I, C), has no known pyrogenic or toxic effects even at high dosages (6). Both poly(I, C) and poly(A, U) also stimulate immune responses and in recent studies, principally with poly(A, U), we provided evidence indicating that such effects are due to changes in cAMP-mediated events in immunocompetent cells (4, 5, 19, 20). Poly(A, U) was recognized as an enhancer of adenylyl cyclase activity (32), i.e., of the enzyme responsible for cAMP formation, and it was shown that stabilizers of cAMP levels, namely theophylline or caffeine, can magnify the effects of poly(A, U) on antibody formation (5). Since immune responses are important in the control of tumor growth (30) we have now explored the ability of immunoenhancing poly(A, U), poly(I, C), and/or theophylline to affect the intradermal growth of RLV cells in syngeneic hosts.

This report will summarize data that indicate a degree of antitumor activity of poly(A, U), poly(I, C), and theophylline, and it will provide evidence that such effects are not necessarily the result of modified host responses but instead can be attributed to a large extent to a direct suppressive effect on the neoplastic cells.

MATERIALS AND METHODS

Tumor Cell Line. The RLV tumor cell line (MCDV-12), induced by Rauscher leukemia virus, was maintained by serial i.p. passage through BALB/c mice, transfers being made every 7 days. For experimental use, tumor cells were removed from the peritoneal cavity of mice that had received the passage tumor 5 to 7 days previously. The cells were harvested by i.p. injection of 2 ml of MEM and subsequent aspiration of 2 ml from the peritoneal cavity. The cells were centrifuged, washed twice in cold MEM, counted, and then resuspended in an appropriate volume of cold MEM. Some changes in the properties of this line (rate of growth, responses to treatment) were noted toward the end of the experimental period.

Animals. BALB/c female mice, 6 to 10 weeks old, obtained from Cumberland View Farms (Clinton, Tenn.), were used in tests with the RLV tumor cells.

Tumor Implantation. Tumor cells (0.02 ml) were injected intradermally, with a 1-ml tuberculin syringe and 27-gauge needle, into the shaved flank of BALB/c recipients. Dose levels of 10^3, 10^4, or 10^6 cells/animal were used. In large experiments, where the time intervals between the collection of tumor cells and their implantation differed, particularly among the groups given injections first and last, standing of the cells in suspension sometimes resulted in clumping and an occasional reduction of the subsequent rate of tumor growth in vivo. For avoidance of such artifacts, a randomization procedure was adopted in all of the more recent tests. All animals of a given experiment were first implanted with tumor cells and then separated at random into treatment groups. Such randomization, while overcoming the effects of differential time of implantation after tumor cell collection, confirmed the basic validity of the data to be reported.

Measurement of Tumor Development. Tumor growth, in terms of the average area (sq mm) of palpable tumors, was

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measured daily with calipers to determine the diameters. No measurement of tumor depth was attempted. Each experimental group always consisted of 4 to 5 animals, and the average tumor area for a group was calculated as the arithmetic mean.

**Treatment Procedures and Materials.** For *in vivo* treatment, the agents used, poly(A, U) and poly(I, C) (Miles Laboratories, Elkhart, Ind.), as well as aminophylene (= ethylenediamine • theophylline; Sigma Chemical Co., St. Louis, Mo.), were injected i.p. in 0.2 ml sterile, nonpyrogenic 0.85% NaCl solution. For "pretreatment," MEM-washed ascites tumor cells were exposed to the agents named above for 30 min at 37° in serum-free MEM, then washed once in cold MEM, and implanted as usual.

**Irradiation.** BALB/c mice were irradiated with a total of 850 rads, 100/min, prior to the implantation of tumor cells, with the use of a Norelco Mg-150 X-ray machine, at 12 ma and 150 kV with a 1-mm aluminum filter.

**RESULTS**

With intradermal inocula containing either 10³, 10⁴, or 10⁶ RLV tumor cells, retardation of tumor development, particularly during the early period of tumor growth, occurred in each of a large series of experiments designed to test the effect of poly(A, U), poly(I, C), and/or theophylline. Representative data are shown in Charts 1 and 2 and in Table 1. Treatment, however, did not prevent death, presumably caused by generalized viremia, which occurred 8 to 17 days after tumor cell implantation, the time being directly proportional to the size of the tumor inoculum.

Poly(I, C) consistently proved to be more effective in retarding tumor growth than poly(A, U). Nevertheless, we chose to emphasize studies with poly(A, U) in view of the fact that this double-stranded polynucleotide is nontoxic, nonpyrogenic, and a poor interferon inducer; whereas poly(I, C) is toxic, pyrogenic, and a good interferon inducer.

It was consistently observed that optimal effects were dependent on appropriate polynucleotide dosage and times of treatment. Thus, as indicated in Chart 1, a 300 µg dose of poly(A, U) per injection was always more effective than twice this amount; similarly, 300 µg poly(I, C) per injection gave as good or better results than 600 µg and about the same results as 150 µg/injection. As illustrated in Chart 1, with the same dose per injection, 3 sequential injections, on Days -1, 0, +1, gave better effects than 2 sequential injections on Days 0 and +1. However, when the total dose injected was the same and

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**Table 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Average tumor size (sq mm) on Day 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated controls</td>
<td>66 ± 10.0²</td>
</tr>
<tr>
<td>Poly(A, U) (300 µg)</td>
<td>19 ± 2.3</td>
</tr>
<tr>
<td>Theophylline (200 µg)</td>
<td>20 ± 9.4</td>
</tr>
<tr>
<td>Poly(I, C) (300 µg)</td>
<td>21 ± 9.5</td>
</tr>
<tr>
<td>Poly(I, C) (300 µg) + theophylline</td>
<td>50 ± 3.7</td>
</tr>
<tr>
<td>Poly I, C (100 µg) + theophylline</td>
<td>30 ± 6.1</td>
</tr>
<tr>
<td>Poly(I, C) (30 µg) + theophylline</td>
<td>11 ± 3.7</td>
</tr>
</tbody>
</table>

² Mean ± S.E.
Effects of poly(I, C) and poly(A, U) on the growth of RLV tumors in irradiated and nonirradiated BALB/c mice

The polynucleotides were injected, 450 μg/injection, on Days 0 and 1 in relation to intradermal implantation of 10⁶ tumor cells. Each group contained 4 animals.

<table>
<thead>
<tr>
<th>Group</th>
<th>Average tumor size (sq mm) on Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(I, C)</td>
<td></td>
</tr>
<tr>
<td>Nonirradiated</td>
<td>32 ± 7.8</td>
</tr>
<tr>
<td>Irradiated</td>
<td>35 ± 2.0</td>
</tr>
<tr>
<td>Poly(A, U)</td>
<td></td>
</tr>
<tr>
<td>Nonirradiated</td>
<td>58 ± 13.5</td>
</tr>
<tr>
<td>Irradiated</td>
<td>54 ± 11.1</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
</tr>
<tr>
<td>Nonirradiated</td>
<td>95 ± 6.2</td>
</tr>
<tr>
<td>Irradiated</td>
<td>82 ± 8.0</td>
</tr>
</tbody>
</table>

*Mean ± S.E.*

distributed equally in several injections, the effect was not dependent on the number of injections. Thus, 2 injections of poly(A, U), 450 μg/injection, on Days 0 and +1, were essentially as effective as 3 injections of 300 μg each on Days −1, 0, and +1 or on Days 0, +1, and +6. Prolonged treatment, spaced at 48-hr intervals, was no more effective than the initial short-term treatment, and prolonged treatments at 24-hr intervals, 300 μg poly(A, U) on Days −1 through +4, always gave poorer results than 2 or 3 initial treatments. Furthermore combinations of poly(A, U) and poly(I, C), with optimal dose levels and injection schedules, did not result in any greater effects than that produced by the most effective component, i.e., poly(I, C) alone.

In view of the findings that phosphodiesterase inhibitors, such as theophylline and caffeine, potentiate the effectiveness of polynucleotides in stimulating immune responses, the effect of combining poly(A, U) and theophylline was tested. A series of tests, of which Chart 3 is representative, showed that theophylline potentiated the antitumor effects of poly(A, U). Theophylline alone, which had little or no effect on immune responses (5), also had a retarding effect on the growth of the syngeneic tumor cells (Charts 2 and 3). Theophylline was effective when used in combination with a low dose of poly(I, C) (30 μg/injection); however, when used in combination with higher doses of poly(I, C), unfavorable results were obtained (Table 1), the reduced effectiveness being a function of the dose of poly(I, C).

Up to this point we proceeded in the belief that the retarding effects of polynucleotides and of theophylline might be due to alterations of appropriate immune responses of the host. This belief had to be modified by our finding that the polynucleotides were essentially as effective in irradiated animals (Table 2) which had received 800 rads prior to tumor implantation, i.e., a radiation dose that is known to reduce immune responses substantially. Therefore the effects of direct exposure of the tumor cells to polynucleotides or theophylline, prior to implantation into syngeneic hosts, were determined. The dosages used [0.001 μg poly(A, U) or theophylline per ml] were chosen on the basis of the effectiveness of such concentrations on antibody formation and lymphocyte transformation in mouse spleen cell cultures (19, 32). Inhibitory effects of preexposure of the tumor cells to poly(A, U), theophylline, or a combination thereof were observed in the majority of the tests conducted (4 of 7). One such result is illustrated in Chart 4. No explanation has yet been found for the lack of effect of pretreatment in some of the tests, but this variability might be related to the physiological state of the tumor cells at the time of implantation since tumor cells harvested after 5 days of in vivo passage appeared to be less susceptible to the in vitro treatment than tumor cells harvested after 7 days.
DISCUSSION

It is evident from the data obtained that the rate of growth of the RLV tumor cell line in syngeneic BALB/c mice is retarded by poly(A, U), poly(I, C), and theophylline. When appropriate dosage levels are used, this retardation tends to be more pronounced when treatment consists of a combination of polynucleotides + theophylline than when either substance alone is used. The effects may appear far from spectacular when viewed in terms of differences in tumor size. However, when one considers that, particularly during the early period of tumor growth, a difference in a few mm in tumor size can correspond to a difference of at least 1 log in the initial number of viable tumor cells, as revealed by titrations of dose responses to the intradermal implantation of RLV cells, the magnitude of the effects of the drugs becomes more impressive. Furthermore, one can suspect that such effects might become even more significant when they are combined with the effects of other antitumor agents.

We have also observed antitumor effects of poly(A, U) and theophylline in preliminary trials with an intradermally injected RLV-induced ascites tumor cell line in CDF1 mice, with a Moloney ascites tumor cell line in CDF1 mice, and with a suspension of cells injected s.c. from a Graffi solid tumor transplant in syngeneic C57BL mice (3). However, recalling the description in a recent report regarding the variability of effectiveness of poly(I, C) in different tumor systems (11), we too noted that the magnitude of poly(A, U) and theophylline effects differs with different test systems, a probable reflection of the complexity of factors that can contribute to the growth of virus-induced tumors.

In previous studies (7) we observed an inhibitory effect of poly(A, U) on the frequency of regrowth of tumors in C57BL mice into which pieces of a MC-induced tumor had been implanted s.c. and had then been excised 4 days later, prior to the initiation of treatment. We obtained similar poly(A, U) effects with another, independently derived, MC-induced tumor cell line but not with a 3rd MC-induced tumor cell line, which, however, proved to be susceptible to treatment with theophylline or poly(A, U) as well as chlorphenesin [3-(p-chlorophenoxo)-1,2-propanediol]. The latter is an agent that modifies immune responses (2), stimulates adenyly cyclase activity (27), and retards the growth of RLV tumor cells, as well as that of a number of other transplantable tumors (H. J. Spencer, unpublished data).

At the moment it is difficult to explain the apparent variability in responses to poly(A, U) and theophylline treatment by different cell lines, just as it is difficult to account for the fact that positive results with a given cell line are dependent on appropriate dosages and timing, excessive treatment being detrimental. It appears that both the physiological state and the genotype of the cells may influence the degree of effectiveness of these agents. The contribution of the physiological state is indicated by differences in results when cells harvested from donors inoculated i.p. 5 or 7 days previously were used as inoculum; in general, the 7-day-old cells proved to be somewhat more susceptible to treatment than the 5-day-old cells. An influence of the genotype is suggested not only by the differences in responsiveness of different tumor cell lines but also by the lessened susceptibility of the RLV-induced tumor cell line in recent tests compared to earlier tests with the same cell line.

These studies were initiated with the expectation that the antitumor effects of polynucleotides and of theophylline may be mediated by the known effects of these agents on immune responses (4, 5, 19, 20). However, the results obtained in tests with irradiated animals and after direct treatment of the tumor cells prior to implantation suggest that, at least during the early period of tumor development scrutinized in the present trials, a substantial portion of the effects of the polynucleotides and theophylline represents a direct effect on the neoplastic cells and may be independent of the known capacity of these agents to alter immune responses. The conclusion that direct effects are involved must be limited for the time being to the RLV (MCDV-12) line, since similar tests have not yet been conducted with the other tumor cell lines that are retarded in their growth following in vivo treatment. However, direct effects of poly(I, C) on macromolecular biosynthesis in 3 different murine tumors have been reported previously by Levy and Riley (26).

Others (15, 20, 22) have recently reported inhibitory effects of cAMP or dibutyryl-cAMP on tumor cells in vitro, and Johnson et al. (21) as well as Hsie and Puck (18) have observed a restoration of normal phenotypic characteristics in neoplastic cells exposed to cAMP (cf. Ref. 28). There is currently a strong suspicion that the synthesis of macromolecules, particularly of nucleic acids, may be regulated by cAMP-mediated events and that there may be important differences in the cAMP system between normal and neoplastic cells (8—10). In studies on the regulation of immune responses, we have collected evidence (4, 5, 19, 20) which indicates that the effects of polynucleotides and of phosphodiesterase inhibitors, such as theophylline, involve a cAMP-mediated amplification system in immunocompetent cells, and we have shown (Ref. 32, and more recent unpublished data) that the double-stranded synthetic polynucleotides stimulate the activity of adenyl cyclase in mouse lymphocytes. It is therefore tempting to ascribe the inhibitory effects of poly(A, U) and theophylline on neoplastic cells to alterations in the endogenous cAMP system of the cells and to relate them to the inhibitory effects of exogenous cAMP noted by others. However, direct data documenting such a suspected relationship are as yet lacking.

In the activation of immunocompetent cells, depending on the intensity of the effects of exogenous modifiers on the endogenous cAMP system, cellular responses can be either enhanced or reduced (4); whereas the functions of already activated cells, e.g., the release of effectors from sensitized cells after exposure to antigen, apparently can be reduced only by exogenous cAMP or by modifiers of endogenous cAMP levels (16). Furthermore, we have recently observed that interactions between allogeneic spleen lymphocytes result in an elevation of adenyly cyclase activity and that endogenous cAMP levels rise rapidly, in both tumor and spleen cells, when RLV-induced tumor cells are mixed in vitro with syngeneic
normal spleen cells (C. Shiozawa and W. Braun, unpublished data). Such observations raise the interesting question of whether the appearance of new antigenic determinants on the surface of transformed cells [which, as previously noted by others (31), can lead to interactions reminiscent of mixed lymphocyte reactions between tumor cells and autochthonous lymphocytes] may contribute to an alteration in endogenous cAMP levels and a triggering of proliferative events. Once activated, proliferating tumor cells might display only inhibited responses following any additional alteration of endogenous cAMP levels, as has been noted in activated immunocompetent cells. Moreover, it is conceivable that basic inhibited responses following any additional alteration of activated, proliferating tumor cells might display only lymphocyte reactions between tumor cells and autochthonous cells from different tumor lines and in cells of different physiological state might account, in part, for the variability that has been noted by us and others in regard to the effectiveness of exogenous agents with the potential of exerting effects on the endogenous cAMP system.

The fact that 3 agents with known influence on the formation and degradation of endogenous cAMP, namely, synthetic polynucleotides, chlorphenesin, and theophylline, display a degree of antitumor activity is unlikely to be fortuitous. There is yet another observation that supports the idea that modifiers of the endogenous cAMP system may alter properties of neoplastic cells as effectively as exogenous cAMP. Interferon has antitumor activity (13), and it was recently observed (W. Braun, C. Shiozawa, and H. Levy, unpublished data) that interferon preparations contain a factor that elevates adenyl cyclase activity of spleen lymphocytes and enhances antibody formation. Thus the antitumor activity of interferon preparations may also be attributable to effects on the cAMP system, but this interpretation, as well as the suggestion that the apparent direct antitumor effects of polynucleotides and theophylline may operate via cAMP-mediated events, will require appropriate experimental confirmation.

ACKNOWLEDGMENTS

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