Inhibition of C3H/He Mouse Mammary Tumor Growth by Combined Treatment with Cyclophosphamide and Polyadenylc-Polyuridylic Acid

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ABSTRACT

The antitumor effect of an immunomodulator, polyadenylc-polyuridylic acid, in combination with cyclophosphamide (CY) was studied in C3H/He mice bearing established mammary tumors. On Day 14 after tumor graft, mice received either CY (90 mg/kg) alone every 2 weeks for a total of four inoculations or alternate weekly inoculations of the same dose of CY and polyadenylc-polyuridylic acid (300 μg) or Bacillus Calmette-Guérin (400 μg) during 8 consecutive weeks. On Day 170, the following results were obtained. (a) Mice receiving CY alone showed significantly retarded tumor growth; nevertheless, 30 mice of 34 (88%) died of tumor, and only 1 mouse (3%) was tumor free. (b) In mice receiving combined CY and Bacillus Calmette-Guérin, no more significant tumor inhibition was observed than those receiving CY alone. (c) The most significant tumor inhibition was observed in mice receiving combined CY and polyadenylc-polyuridylic acid. Average tumor diameter on Day 63 was one-third (2 mm) of that of mice receiving CY alone (7 mm); 25 mice of 44 (57%) died of tumor; and 11 mice (25%) were tumor free.

INTRODUCTION

An essential requirement for successful immunotherapy in cancers would be the least tumor burden of hosts. Administration of immunostimulants alone into hosts bearing already established tumors has proven to be ineffective to inhibit tumor growth (2). In such a situation, the rational approach would be the use of potent immunostimulants in combination with surgery or with cytocidal chemotherapeutic agents, hence allowing stimulated host immune mechanism by the former to react more efficiently against residual tumor cells.

In most works on nonspecific immunotherapy of cancers reported until now, living bacteria or bacterial products were used as adjuvants to conventional surgery or chemotherapy (9, 22). Encouraging results were reported; however, these bacterial adjuvants could provoke occasionally undesirable complications (27), even, in some situations enhancing effects on tumor growth (21).

Double-stranded complex of the synthetic polyribonucleo-

dides poly(A)-poly(U)3 has proven to be a highly potent immunostimulator to both humoral and cell-mediated immune responses (3, 13). This substance, devoid of any toxic or enhancing effects, has been successfully used as an adjunct to surgery in spontaneous mammary tumors of mice and in transplantable melanoma of hamsters (17). Prophylactic effects of the agent against spontaneous mammary carcinoma and spontaneous leukemia in mice have been described (8, 15).

Recently, in a study on clinical application of poly(A)-poly(U) to operable breast cancers in our institute, it was revealed that overall survival was significantly higher in patients treated with this agent following surgery, in whom 5 years relapse-free survival was also increased (16).

In the present paper, we report our findings on synergistic effect on tumor inhibition following sequential administration of CY in combination with poly(A)-poly(U) into C3H/He mice bearing an established transplantable mammary tumor. Mice receiving the combined treatment showed significantly more retarded tumor growth, lower mortality, and higher rate of complete tumor regression than did those treated with either agent alone.

MATERIALS AND METHODS

Mice. Inbred female C3H/He mice, 4 to 6 weeks of age, were supplied by the Animal Selection Center, Orléans, France.

Tumor Cells. A transplantable tumor cell line (TM1), developed in our laboratory from a spontaneous mammary tumor of a C3H/He mouse (18), was used for animal inoculation to produce s.c. tumors.

YAC-1, a tissue culture cell line of a Moloney virus-induced lymphoma of A/Sn origin (5), was used as target cells in in vitro cytotoxicity assays. Both cell lines were routinely cultivated in Roswell Park Memorial Institute Tissue Culture Medium 1640 (GIBCO Bio-Cult Ltd., Paisley, Scotland) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml) (hereafter referred to as complete medium). For YAC-1 culture, this medium was further supplemented with 1 mM sodium pyruvate and 5 x 10^{-3} M 2-mercaptoethanol.

CY. CY (Endoxan-Astra; Laboratories Lucien, Colombes, France) was dissolved in sterile distilled water and immediately administered i.p. in doses of 90 mg/kg body weight.

BCG. Lyophilized Pasteur strain of BCG (Vaccine BCG Pasteur scarification; Institut Pasteur, Paris, France) was dissolved in 0.15 M NaCl and inoculated i.p. in doses of 400 μg (approximately 3.4 x 10^6 viable units).

poly(A)-poly(U). poly(A)-poly(U) was prepared from ADP and UDP by Dr. M. Michelson (Institut de Biologie Physico-Chimique, Paris, France). The respective monomers were polymerized with polynucleotide phosphorylase, and the purified polynucleotides were mixed in a

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References


1 The abbreviations used are: poly(A)-poly(U), polyadenylc-polyuridylic acid; CY, cyclophosphamide; BCG, Bacillus Calmette-Guérin; NK, natural killer; SNM, splenic nonadherent mononuclear.

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Experimental Design. Adult female C3H/He mice were inoculated s.c. on right flank with 10^6 TM1 tumor cells, a dose which gave 100% tumor takes in normal syngeneic adult mice. Two weeks later (Day 14), mice bearing approximately equivalent size of palpable tumors (average, 3 mm in diameter) were selected out and randomized into 6 groups: Group I, control; Group II, BCG alone (every 2 weeks for a total of 4 inoculations from Day 21); Group III, poly(A)-poly(U) alone (similar to Group II); Group IV, CY alone (every 2 weeks for a total of 4 inoculations from Day 14); Group V, CY plus BCG (alternate weekly inoculations from Day 14 during 8 consecutive weeks); Group VI, CY plus poly(A)-poly(U) (similar to Group V).

Tumors were measured twice weekly with caliper, and average diameters were calculated from measurements in 2 planes. All mice were weighed before measuring tumor size each time.

In vitro Cytotoxicity Assay. YAC-1 cells, suspended at 2 x 10^6 cells in 0.2 ml complete medium, were incubated at 37°C in a water bath for 1 hr with 200 μCi Na_2^51CrO_4 (specific activity, 1 mcI/ml; NE2-030S; New England Nuclear, Boston, Mass.). After washing, tumor cells were resuspended in 1 ml complete medium and kept for 1 hr at 4°C. By this procedure, spontaneous ^51Cr release could be significantly reduced. The labeled tumor cell suspension was centrifuged and resuspended in complete medium, and 10^6 viable cells in 0.2 ml volume were distributed with the aid of an Eppendorf pipet in the bottom of each plastic tube (75 x 13 mm). Effector cells were prepared from spleens of mice sacrificed by neck elongation. Spleens were perfused through their longitudinal axis with 5 ml complete medium using a hypodermic needle (12 x 0.4 mm) to eliminate RBC and minced with curved scissors, and monodispersed cell suspension was collected after decantation for 1 min. After washing to eliminate adherent cells which could eventually suppress the effector phase of NK activity (24, 25), cells were resuspended in complete medium and incubated in Falcon plastic Petri dishes (Falcon No. 3002; Falcon Plastics, Oxnard, Calif.) for 1 hr at 37°C in 5% CO_2 humidified incubator. Nonadherent spleen cells were harvested, and 10^6 mononuclear cells in 0.2 ml culture medium were added in each tube (the ratio of target to effector cells was 1:130). Target cells were also added to replicate tubes containing 0.2 ml medium alone to determine spontaneous ^51Cr release and to tubes containing 0.2 ml medium plus detergent (0.25% Triton X-100; Sigma Chemical Co.) to determine maximal release. Five replicates were made for each assay. The tubes were incubated for 4 hr at 37°C in 5% CO_2 humidified incubator. After centrifugation, aliquots of 0.1 ml supernatant were carefully removed and counted in a Gamma counter (Gamma 4000; Beckman). The percentage of cytotoxicity was calculated by the following formula:

\[
\text{% of cytotoxicity} = \frac{\text{test release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100
\]

Spontaneous release of ^51Cr from YAC-1 targets in medium alone ranged from 7 to 15% of the maximal release.

Statistical Analyses. Mean tumor diameters and percentage of cytotoxicity were analysed by Student’s t test. Survival curves of different groups of tumor-bearing mice were established by the method of Kaplan and Meier (14), and the differences between groups were calculated by log-rank test (20). Rate of complete tumor regression and mortality were compared by means of the χ-square test.

RESULTS

Effect of CY and/or poly(A)-poly(U) or BCG on Growth of TM1 Tumor. The tumor growth of the 6 groups of mice on Day 63 after tumor graft is shown in Table 1. Both control untreated tumor-bearing mice (Group I) and those treated with poly(A)-poly(U) alone (Group III) developed relatively rapid growth of tumors whose mean diameters attained 12.8 and 12.5 mm, respectively. Thus, the administration of poly(A)-poly(U) alone did not inhibit nor enhance the tumor growth. Mice treated similarly with BCG alone (Group II) showed accelerated tumor growth, and their mean tumor diameter was significantly increased (16.1 mm) if compared with those of the above 2 groups. Mice treated with CY alone (Group IV) and those treated with CY plus BCG (Group V) showed obviously slower tumor growth than the above 3 groups. Meanwhile, the evolutions of the tumor growth of these 2 groups (Groups IV and V) were quite similar, and the differences in tumor size were not significant at any point of observation. However, mice treated with CY plus poly(A)-poly(U) showed entirely inhibited pattern of tumor growth, and their mean tumor diameters never exceeded 2.2 mm in the whole period of observation. The differences in tumor size between Groups VI and IV or V were highly significant (p < 0.001).

At the end of experiments (Day 170), the mortality by tumors and the rate of complete tumor regression in the 6 groups were compared. As shown in Table 1, all mice in Group I (no treatment), Group II (BCG alone), and Group III (poly(A)-poly(U) alone), except 3 mice in Group I, died of tumor, whereas in the remaining groups the number of mice that died of tumor were 30 out of 34 (88%) in Group IV (CY alone), 24 out of 30 (80%) in Group V (CY + BCG), and 25 out of 44 (57%) in Group VI (CY + poly(A)-poly(U)), respectively. The difference in mortality between Groups VI and IV or V was highly significant (p < 0.001). The rate of complete tumor regression was

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of total mice</th>
<th>Tumor size on Day 63 (mm)</th>
<th>Died of Tumor</th>
<th>Survivors</th>
<th>Tumor free</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>40</td>
<td>12.8 ± 6.97^a</td>
<td>37 (92.5)^b</td>
<td>3 (7.5)</td>
<td>1 (2.5)</td>
</tr>
<tr>
<td>II</td>
<td>BCG alone</td>
<td>39</td>
<td>16.1 ± 2.50</td>
<td>39 (100)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>poly(A)-poly(U) alone</td>
<td>19</td>
<td>12.5 ± 6.51</td>
<td>19 (100)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IV</td>
<td>CY alone</td>
<td>34</td>
<td>6.6 ± 6.01</td>
<td>30 (88)</td>
<td>4 (12)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>V</td>
<td>CY + BCG</td>
<td>30</td>
<td>5.7 ± 4.43</td>
<td>24 (80)</td>
<td>6 (20)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>VI</td>
<td>CY + poly(A)-poly(U)</td>
<td>44</td>
<td>2.2 ± 3.71^c</td>
<td>25 (57)^d</td>
<td>19 (43)^e</td>
<td>11 (25)</td>
</tr>
</tbody>
</table>

^a Mean ± S.D.
^b Numbers in parentheses, percentage.
^c p < 0.001 between Groups VI and IV or V.
^d p < 0.001 between Groups VI and IV or V.
^e p < 0.01 between Groups VI and IV.
^f p < 0.01 between Groups VI and IV.
also significantly (p < 0.01) increased in the latter group: 11 mice out of 44 (25%) in Group VI and 1 mouse out of 34 (3%) in Group IV.

Overall survival curves of mice treated with CY and/or poly(A)-poly(U) or BCG were drawn in Chart 1 using the methods of Kaplan and Meier (14). Statistical analyses by log-rank test (20) revealed that the differences in survival rate between Group VI and other groups were significant.

In an attempt to ameliorate these findings, we have performed a series of another experiment in which 2 different doses, 300 and 600 µg, of poly(A)-poly(U) were administered into the tumor-bearing mice in association with CY following the same experimental protocol. Essentially, we have obtained the following results. On Day 65, the mean tumor diameters were 2.7 mm in Group A [CY + 300 µg poly(A)-poly(U)], 2.3 mm in Group B [CY + 600 µg poly(A)-poly(U)], and 6.1 mm in Group C (CY alone), respectively. At the end of experiment (Day 170), the mortality and the rate of tumor-free survivors were as follows: 10 mice of 19 (53%) in Group A, 9 mice of 19 (47%) in Group B, and 14 mice of 20 (70%) in Group C died of tumor, respectively; and 4 mice (21%) in Group A, 5 mice (26%) in Group B, and 1 mouse (5%) in Group C survived without tumor. Thus, as a whole, with both doses of poly(A)-poly(U), significant synergistic tumor-inhibitory effects that are comparable to those observed in the previous experiments were obtained, and little difference on the tumor inhibitory effect between the 2 doses was observed.

**In Vitro Cytotoxicity of Splenic Mononuclear Cells of Mice Inoculated with poly(A)-poly(U) or CY**. Two-month-old female C3H/He mice were randomized into 5 groups, each comprised of 12 mice. They were inoculated i.v. with different doses of poly(A)-poly(U) as follows: Group 1, 0.9% NaCl solution control; Groups 2, 3, 4 and 5, 10, 100, 300, and 600 µg of poly(A)-poly(U), respectively. At 24, 48, 72, and 96 hr after inoculation, 3 mice of each group were sacrificed, and their spleens were removed. Pooled SNM cell suspensions were prepared and submitted to in vitro cytotoxicity assay as described in "Material and Methods."

The results of a representative experiment are shown in Chart 2. The SNM cells of mice inoculated with the 4 different
doses of poly(A)-poly(U) showed elevated cytotoxic activities against YAC-1 targets (except for mice which received 100 μg) significantly at 24 hr and highly significantly (p < 0.001) with dose-dependent pattern at 48 hr after inoculation if compared with those of control mice which received the same volume of 0.9% NaCl solution.

Similar experiments were performed with CY. Two groups of 2-month-old female C3H/He mice were inoculated i.p. with CY (90 mg/kg) and with the same volume of distilled water for control group. At 24, 48, 72, and 96 hr after inoculation, 3 mice of each group were sacrificed, and their spleens were removed for in vitro cytotoxicity assay. The results of a representative experiment are shown in Chart 3. The SNM cells of mice inoculated with CY showed significantly decreased cytotoxicity activities against YAC-1 targets at 48 and 96 hr after CY inoculation.

The next experiments were carried out using TM1 tumor-bearing C3H/He mice inoculated sequentially with CY (90 mg/kg, i.p.) and/or poly(A)-poly(U) (300 μg i.v.) following the same protocol used for the previous tumor inhibition experiments. At the different times in the course of the treatment after tumor graft, 3 to 5 mice of each group were sacrificed, and in vitro cytotoxic activities of SNM cells of these mice were measured as described above.

The results of a representative experiment are shown in Chart 4. On Day 16, SNM cells of mice inoculated once with CY on Day 14 (Group III) showed significantly decreased cytotoxic activity as compared with those of either untreated tumor-bearing mice (Group II) or age- and sex-matched normal mice (Group I). Three weeks later, on Day 37, SNM cells of mice in Groups I and II and those in Group III which were inoculated twice with CY alone on Days 14 and 28 showed almost equally a moderate degree of cytotoxic activity (24 to 28%), whereas SNM cells of mice inoculated twice with poly(A)-poly(U) alone on Days 21 and 35 (Group IV) and those of mice inoculated in the same way with CY and poly(A)-poly(U) (Group V) showed highly significantly augmented activities, and this augmentation was much more pronounced in the latter group (p < 0.001 between Groups IV and V). On Day 44, when the age of mice attained 10 weeks, SNM cells of mice in Groups I, II, III, and IV showed relatively low levels of cytotoxicity suggesting the age-related decline of NK activity (11); nevertheless, those of mice in Group V receiving the combined treatment showed still highly augmented cytotoxic activity (46%).

**DISCUSSION**

We have reported in this paper that sequential administration of CY in combination with poly(A)-poly(U) into mice bearing an established mammary tumor resulted in a significant synergistic inhibition of tumor growth characterized by more retarded...
tumor growth, lower mortality, and higher rate of complete tumor regression than those resulting from either agent alone.

The tumor-bearing mice treated with CY alone in our experiments showed relatively high mortality (88%) and a low rate of complete tumor regression (3%) at the end of experiments. The choice of our regimen for CY administration was made after our preliminary pilot experiments (data not shown), in which inoculation of the mice with CY (90 mg/kg) weekly for 4 weeks instead of every 2 weeks for a total of 4 inoculations or with CY (120 mg/kg) every 2 weeks for a total of 4 inoculations did not ameliorate the above results but caused in both protocols overt toxic symptoms characterized by loss of body weight and early death in about 20% of the mice.

The administration of poly(A)-poly(U) alone into the tumor-bearing mice did not result in any tumor-inhibitory effect in our experiments. It is not unexpected since the injections of poly(A)-poly(U) were started from Day 21 when the mice carried already fully developed tumors. Tumor size at the moment of administration of immunostimulants rather than the doses of the latter seemed to be critical to obtain significant inhibitory effect (2).

We have compared the tumor inhibitory effect of poly(A)-poly(U) with that of BCG when administered in association with CY. No more significant retardation of tumor growth was observed in mice treated with CY plus BCG than those treated with CY alone. Even more, mice treated with BCG alone showed significantly enhanced tumor growth during 2 months of the treatments, which confirms our earlier findings (28) as well as those of others (1, 21). In contrast, in mice treated similarly with CY plus poly(A)-poly(U), their tumor growth was completely inhibited, the mortality was significantly decreased, and the rate of complete tumor regression was also significantly increased.

The mechanisms by which the combined administration of CY and poly(A)-poly(U) resulted in a synergistic tumor inhibition is not clear. Possible involvement of NK cells in immune surveillance against tumors has been suggested (10). In our experiments, poly(A)-poly(U) increased cytotoxic activity of SNM cells against NK-sensitive YAC-1 targets significantly when inoculated into either normal young adult or TM1 tumor-bearing mice. On the contrary, CY caused a reversed effect in the same experimental condition. Depressed NK activity in mice inoculated with CY was reported (23). When the tumor-bearing mice were inoculated sequentially with CY and poly(A)-poly(U) following the same doses and protocol used for the tumor inhibition experiments, their SNM cells showed highly augmented cytotoxic activity after 2 cycles of the treatment if compared to those of mice inoculated in the same way with either agent alone. Thus, repeated administrations of poly(A)-poly(U) into the CY-treated tumor-bearing mice resulted in a synergistic augmentation of SNM cell-mediated cytotoxic activity. Efferocytosis in these tests were of NK nature since they were nonadherent and nonphagocytic cells lacking surface markers characteristic of mature T- and B-cells (data not shown). Such synergistic NK activation by the combined treatment might be one of the important mechanisms governing in vivo synergistic tumor inhibitory effect observed in our experiments.

Precise mechanism through which poly(A)-poly(U) augment NK activity in mice is unknown. It is widely accepted that interferon or interferon inducers augment NK activity both in vivo and in vitro (6, 19). Accordingly, in a recent work (12), mice injected with poly(A)-poly(U) (2 to 10 mg/kg) showed detectable levels of interferon in their plasma. Also, in operated breast cancer patients treated with a lower dose (0.5 mg/kg) of the agent in our institute, activity of their plasma protein kinase with a molecular weight of 72,000, a marker of interferon, was significantly increased.

It was also described that poly(A)-poly(U), when injected into A/Sn mice, greatly increased the number of T-lymphocytes (7). On the other hand, selective depression of suppressor T-cells for delayed-type hypersensitivity in mice inoculated with CY was reported (26).

Taken together, it can be assumed that the synergistic tumor inhibition obtained by the combined treatment might be resulted through the following cumulative events: (a) diminished tumor burden and depressed suppressor cells for delayed-type hypersensitivity caused by CY administration (26); and (b) increased specific T-cell-mediated immune response (7, 13) as well as increased NK activity against residual tumor cells by poly(A)-poly(U) administration.

Based on our experimental data reported in this paper, further clinical application of such combined regimen can be envisaged for the treatment of cancers.

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