Dextran Derivatives in Single and Combination Chemotherapy against Transplantable Mouse Ascites and Solid Tumors

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SUMMARY

Dextran, a typical homopolysaccharide without antitumor activity, was modified by palmitoylation and/or phosphorylation to yield three derivatives: palmitoyldextran phosphate, dextran phosphate, and palmitoyldextran. Of these compounds, only palmitoyldextran phosphate showed growth-inhibitory activity against Ehrlich solid tumor in mice. In combination therapy with mitomycin C, bleomycin, cyclophosphamide, and 5-fluorouracil, palmitoyldextran phosphate manifested strong synergistic effects against both Sarcoma 180 ascites and L1210 leukemic tumors. The compound is not directly cytotoxic against Sarcoma 180 ascites tumor, but it appears to act via activation of peritoneal macrophage. The antitumor activity of palmitoyldextran phosphate apparently is mainly due to immunological host-mediated mechanisms.

INTRODUCTION

A variety of plant polysaccharides possess strong growth-inhibitory properties against several transplantable solid tumors (5, 7, 12, 27). The least toxicity and most marked antitumor effects were observed in a polysaccharide fraction isolated from the leaves of a species of bamboo grass. Previously, Nakahara et al. (16) presented experimental evidence to show that the action of this polysaccharide may be host mediated. In an earlier work, we reported that yeast mannann and glucan are able to exert adjuvant action to enhance the antibody response of mice against implanted solid tumor cells (29).

Since some bacterial constituents, lipopolysaccharides (13, 15), endotoxin (4), glycolipid of mycobacteria (6), and cell wall skeleton of Bacillus Calmette-Guérin (3) are growth inhibitory against some ascites tumors, we investigated the antitumor activity of the dextran derivatives DP, PD, or POP, which possess functional groups similar to those of the bacterial constituents described above.

MATERIALS AND METHODS

Animals. Male dd and DBA/2 × C57BL/6 F1 mice weighing 18 ± 2 g were housed 5/cage in air-conditioned quarters and were provided food and water ad libitum.

Tumors. The tumors used were Sarcoma 180 ascites, Ehrlich solid tumor, and a L1210 line, initially supplied by Dr. Fumiko Fukuoka, National Cancer Center Research Institute of Japan, and maintained in our laboratory in ascites form. dd mice inoculated with 1 × 107 Sarcoma 180 or Ehrlich tumor cells, or DBA/2 × C57BL/6 F1, mice inoculated with 5 × 104 L1210 leukemic tumor cells in our laboratory never spontaneously recovered from the inoculation. Sarcoma 180C, a cell line of Sarcoma 180 ascites tumor, was kindly donated by Dr. Kotaro Koyama of the Research Institute. Tissue culture was incubated at 37° in a moist atmosphere containing 5% CO2.

Chemical Synthesis and Purification of Modified Dextran Pers. Modified dextrans were synthesized according to a previously described method (26). Chemical analysis of DP showed 81.0% glucose by the Molisch method (9) and 18.3% phosphoric acid by the Allen-Nakamura method (17); for PD, 85.6% sugar and 0.6% palmitic acid by gas chromatography; and for POP, 76.8% sugar, 19.0% phosphoric acid, and 0.6% palmitic acid. All synthesized dextran derivatives were free from nitrogen as determined by microelemental analysis.

Assay for Antitumor Activity against Ehrlich Solid Tumor in Vivo. About 1 × 107 cells from a 7-day-old Ehrlich tumor were transplanted s.c. into the right groin. DP, PD, or POP in 0.1 ml of 0.9% NaCl solution was administered i.p. daily for 10 successive days beginning 24 hr after tumor implantation. All the mice were sacrificed 20 days after implantation, and the tumors were extirpated and weighed. Relative tumor growth was calculated from the following formula (7):

\[
\text{Inhibition ratio} \% = \left( \frac{A - B}{A} \right) \times 100
\]

where A is the average tumor weight of the control group and B is the treated group. Ten mice/group were used, and the test was repeated 3 times.

Assay for Antitumor Activity against Ascites Tumor in Vivo. About 1 × 107 Sarcoma 180 ascites tumor cells were implanted i.p. in dd mice, or 5 × 104 L1210 leukemic tumor cells were implanted i.p. in DBA/2 × C57BL/6 F1, mice. A solution of each dextran derivative in 0.1 ml of 0.9% NaCl solution per mouse was administered once daily for 5 successive days beginning 24 hr after tumor implantation. A series of combination therapies was also conducted using these modified dextrans and several conventional antitumor agents such as mitomycin C (Kyowa Hakko Kogyo Co., Tokyo, Japan; Lot MIS-554AE), bleomycin (Nihon Kayaku Co., Tokyo, Japan; Lot B-126), 5-fluorouracil (Kyowa Hakko Kogyo Co., Tokyo, Japan; Lot 5FU194ADD), and cyclophos-
phamide (Shionogi and Co., Osaka, Japan; Lot B210J). Each antitumor agent in 0.1 ml of 0.9% NaCl solution was administered at the indicated dose i.p. once daily for 5 days, 1 hr after injection of dextrans. The dosages of anticancer drugs in combination treatment between the anticancer drugs and dextrans were selected according to the maximum doses previously used separately against each tumor, and which had proven to be an ineffective maximum dose against each tumor. The tumor growth was measured by the total packed cell volume method (22) on the 7th day after tumor inoculation. Life-span elongation was observed for up to 60 days.

**Growth Inhibition in Vitro.** About $1 \times 10^6$ Sarcoma 180C cells/culture tube were incubated in Eagle's minimum essential medium supplemented with 15% calf serum to which was added 1 mg of the dextran derivative per ml. After incubation, the cells were stained with safranin (21) to estimate their viability, which was compared with that of cells incubation, the cells were stained with safranin (20) to estimate their viability, which was compared with that of cells

**Peritoneal Macrophage Cultures.** The methods used were adopted from the method of Ruskin et al. (21). Briefly, peritoneal macrophages were obtained from normal dd mice by washing the peritoneal cavity with 5 ml of Hanks' BSS containing 5 units of heparin per ml. The cell suspension was centrifuged at 800 rpm at 4° for 5 min. The resulting sediment was resuspended in heparinized Hanks' BSS to a concentration of approximately $2 \times 10^6$ cells/ml. Two ml of the cell suspension were placed in Petri dishes (A/S NUNC, Roskilde, Denmark) 3 cm in diameter and incubated for 1 hr. Cells that had not adhered to the dishes during this period were removed by rinsing with Hanks' BSS. The cultures were then reincubated in Medium 199 with Hanks' BSS supplemented with L-glutamine (Grand Island Biological Co., Grand Island, N. Y.) and 10% fetal calf serum.

**Macrophage Cytotoxicity Test.** After 18 hr, the cultures of peritoneal macrophage were challenged with $2 \times 10^5$ Sarcoma 180 ascites tumor cells/ml and/or PPD and/or mitomycin C. After 24 hr of incubation, Sarcoma 180 cells were stained with trypan blue, and the viable cells were counted.

**Plaque-forming Cells.** In order to examine whether dextran and the dextran derivatives accelerated the antibody production against heterologous antigen, the Cunningham and Szenberg (8) modification of the Jerne plaque technique was used. Starting 1 day after Ehrlich solid tumor implantation, dextran or dextran derivatives, 100 mg/kg, were injected i.p. once a day for 10 days into duplicate groups of mice. One, 6, 11, and 16 days after tumor implantation, sheep erythrocytes at a dose of $1 \times 10^8$ cells/0.1 ml of 0.9% NaCl solution were injected into the tail vein. For the combination therapy, starting 1 day after Sarcoma 180 ascites tumor implantation, dextran or dextran derivatives (1 mg/kg) and/or mitomycin C were injected once a day for 5 days into duplicate groups of mice. Two days before and 1 and 4 days after tumor implantation, sheep RBC were injected into the tail vein. Four days after the antigen injection, the spleens were removed from the mice, and the number of plaques formed by the antibody-forming cells per total spleen cells were counted by the conventional method. Each time 5 mice were tested from each experimental group.

**Footpad Tests.** The footpad tests in mice were done according to the method of Katsura et al. (14). Briefly, 100 μg bovine serum albumin and Freund's complete adjuvant were inoculated into the hind flank s.c. The next day, Sarcoma 180 ascites tumor inoculation and drug treatment were as described above. After 5 days, 12.5 μg of alum-precipitated bovine serum albumin in a volume of 0.025 ml were injected into the right hind footpad; a similar volume of diluent was injected into the left hind footpad. Twentyfour hr later, the difference in thickness between the pre- and postinjection foot was recorded and used as a measure of the amount of swelling. Each time, 10 mice were tested from each experimental group.

Student's t test was used to evaluate the significance of the differences between the groups given the test compound and the control group.

**RESULTS**

**Growth-inhibitory Activity of Modified Dextrans against Ehrlich Solid Tumor.** Table 1 shows the effect of dextran and PDP on solid Ehrlich tumor in dd mice at 1 and 100 mg/kg/day for 10 successive days. PDP showed an inhibition ratio of 56% ($p < 0.01$) at 100 mg/kg/day. The other dextran derivatives, DP and PD, were ineffective. Ten mice/group were used, and the test was repeated 3 times.

**Growth-inhibitory Activity of Modified Dextrans against Sarcoma 180 Ascites Tumor.** dd mice were implanted i.p. with $1 \times 10^7$ of the Sarcoma 180 tumor cells. A series of

### Table 1

<table>
<thead>
<tr>
<th>Materials</th>
<th>Dose (mg/kg/day for 10 days)</th>
<th>Av. tumor wt (g)</th>
<th>Inhibition ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
<td>Experiment 2</td>
<td>Experiment 3</td>
</tr>
<tr>
<td>PDP</td>
<td>1</td>
<td>6.8</td>
<td>15.1</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3.0</td>
<td>4.2</td>
</tr>
<tr>
<td>Dextran</td>
<td>1</td>
<td>6.5</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>8.1</td>
<td>11.9</td>
</tr>
<tr>
<td>Control</td>
<td>7.0</td>
<td>10.6</td>
<td>6.8</td>
</tr>
</tbody>
</table>

* Ten mice/group.

* Versus the control group.
combination therapies was examined using PDP (1 mg/kg/day i.p.) and mitomycin C (0.01 mg/kg/day i.p.) once daily for 5 days beginning 24 hr after tumor implantation. The tumor growth was measured by the total packed cell volume method on the 7th day after tumor inoculation. As seen in Table 2, the group receiving combination treatment showed about 91% inhibition of tumor growth.

**PDP-Induced Life-span Elongation of Mice Bearing Sarcoma 180 Ascites or L1210 Leukemic Tumors.** There has been considerable interest in recent years in the use of combinations of drugs against experimental (11) and clinical (24) neoplasia. We tried combination therapy of dextran derivatives with mitomycin C, bleomycin, cyclophosphamide, and 5-fluorouracil. The dextran derivatives and the other antitumor agents were injected i.p. by the method described above. First, the combination therapy of mitomycin C (0.5 mg/kg/day for 5 days) and dextran derivatives (1 mg/kg/day for 5 days) was tested. PDP and DP showed significant synergism when combined with mitomycin C (Chart 1). Ten of the 20 mice treated with PDP and mitomycin C survived for more than 60 days after implantation with noticeable tumor regression at that time. Similarly, 20 mice/group were used, and the following experiments were carried out: a combination therapy of PDP with bleomycin (Chart 2), with cyclophosphamide (Chart 3), and with 5-fluorouracil (Chart 4) against Sarcoma 180 ascites tumor in dd mice, all of which combinations also showed remarkable synergism between the drugs.

**Table 2**

<table>
<thead>
<tr>
<th>Materials</th>
<th>Inhibition ratio (%)</th>
<th>No. of cured/no. of treated mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDP + mitomycin C</td>
<td>91</td>
<td>10/12</td>
</tr>
<tr>
<td>PDP</td>
<td>17</td>
<td>0/12</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>9</td>
<td>0/36</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0/36</td>
</tr>
</tbody>
</table>

The synergistic effect of PDP and mitomycin C against L1210 leukemic tumor in DBA/2 × C57BL/6 F1 mice is shown in Chart 5. Fifty-two mice were implanted i.p. with 5 × 10⁶ L1210 cells and were then divided at random into 4 groups of 13 mice. Twenty-four hr later, a solution of PDP in 0.9% NaCl solution and/or mitomycin C was injected i.p.
tumor cells in vitro. Addition of mitomycin C alone (0.3 mg/ml) and PDP (0.5 mg/ml) showed cytotoxicity to the tumor cells (Table 3).

Effect of Normal Peritoneal Macrophages and PDP with Mitomycin C on the Cytocidal Activity against Sarcoma 180 Ascites Tumor Cells. In the preceding experiment, PDP showed a direct enhancement of mitomycin C toxicity. With this in mind, we then modified our in vitro experimental system by the addition of peritoneal macrophages according to the method of Ruskin et al. (21). Table 4 shows the results of this experiment; the synergistic effect, not shown with mitomycin C (0.1 mg/ml) plus PDP (0.5 mg/ml), without normal peritoneal macrophage (Table 3), is detected upon combination of the same dosages of mitomycin C and PDP. Furthermore, the cytocidal activity against the tumor cells was detected upon PDP and the macrophage, although the activity was still weaker than in combination of PDP with mitomycin C and the macrophage.

Effect of Dextran Derivatives on Antibody Formation against Sheep RBC by the Cunningham Method in Mice Bearing Ehrlich Solid Tumor. Since the antitumor action of PDP was not by direct cytotoxicity but rather by a host-mediated response or by activation of tumor-killing cells, the effect of the PDP on various immune responses was examined in order to further elucidate the mechanism of action of the drug. The plaque-forming test was carried out on tumor-bearing mice to determine whether PDP would increase antibody production in the spleen against sheep erythrocytes. As seen in Table 5, the number of plaque-forming cells in PDP-treated mice 10 days after Ehrlich solid tumor implantation showed a significant difference between those in normal and in dextran-, DP-, and PD-treated mice. The kinetics of antibody formation by spleen cells of PDP-treated mice with Ehrlich solid tumors is shown in Chart 7. The increase in plaque formation was not detectable in normal PDP-treated mice but was detectable in tumor-bearing PDP-treated mice.

Effect of Dextran Derivatives on Antibody Formation against Sheep RBC by the Cunningham Method in Mice Bearing Sarcoma 180 Ascites Tumor. Table 6 shows the plaque formation 5 days after Sarcoma 180 ascites tumor inoculation in mitomycin C and/or dextran derivative-treated mice, and its kinetics is illustrated in Chart 8. On Day 2 a decrease in plaque formation was observed in tumor-bearing mice treated with mitomycin C (0.5 mg/kg) and PDP for 5 successive days. Three of 13 mice treated with PDP and mitomycin C survived for more than 60 days after tumor implantation. These experiments were duplicated, and similar results were obtained. An abdominal incision confirmed the absence of the remaining tumor in those mice that survived for 60 days after tumor implantation.

Direct Cytocidal Action of Dextran Derivatives on Cultured Cells. In order to examine whether PDP has a direct cytotoxic or growth-inhibitory effect on cells in tissue culture, Sarcoma 180C cells were grown in a medium containing PDP (1 mg/ml), and the viability of the cells was examined after 0, 24, 48, and 72 hr of incubation. Viability of the cells cultured in a medium containing PDP was no different from that of the cells cultured without PDP. PDP has neither direct cytotoxicity nor growth-inhibitory activity against the tumor cells in vitro (Chart 6).

Enhancement of Mitomycin C Cytotoxicity against Sarcoma 180 Ascites Tumor Cells by PDP. The above results indicate that PDP is not directly cytotoxic in vitro but does amplify the antitumor activity of several conventional anticancer drugs in vivo. Therefore, we examined the combined effect of mitomycin C and PDP on Sarcoma 180 ascites tumor cells in vitro. Addition of mitomycin C alone (0.3 mg/ml) to the Sarcoma 180 cells had no effect at the end of a 24-hr culture period. On the other hand, the combination of mitomycin C (0.3 mg/ml) and PDP (0.5 mg/ml) showed cytotoxicity to the tumor cells (Table 3).

Antitumor Activity of Dextran Derivatives
Same as did the normal mice, mice, while the combination therapy group responded the Tumor-bearing Mice. To examine the cellular reaction, we conducted a footpad test. As shown in Table 7, the mice bearing ascites tumors responded less than the normal controls; significantly different from that of the other treated groups.

It was reported by Azuma et al. (2) that Bacillus Calmette-Guérin cell wall skeleton, a well-known and potent antitumor agent, is composed of mycolic acid, polysaccharide, mucopeptide, and phosphate. PDP is composed of similar components: fatty acid, polysaccharide, and phosphate.

DP, PD, and PDP did not show growth inhibition against Sarcoma 180 ascites tumor when administered alone, but PDP showed a remarkable synergistic effect against Sarcoma 180 ascites tumor and L1210 leukemic tumor in combination therapy with mitomycin C, bleomycin, cyclophosphamide, and 5-fluorouracil (Charts 1 to 5).

PDP alone did not show growth-inhibitory activity against Sarcoma 180C strain nor did it show direct cytocidal activity against Sarcoma 180 ascites tumor cells in vitro. Both PDP and DP are nontoxic to mice at the dose used in this work; intact mice given i.p. injections of up to 1000 mg/kg of PDP, and DP showed no signs of toxicity. The mechanism of antitumor activity of PDP appears to be an indirect effect. When Sarcoma 180 ascites tumor cells were incubated with PDP and mitomycin C, there was an enhancement of cytotoxicity (Table 3), which may indicate a direct interaction of the drugs. Furthermore, PDP alone activated peritoneal macrophage (Table 4), and this effect was even greater in the presence of mitomycin C.

Alexander and Evans (1, 10) showed the activation of macrophage by endotoxin, double-strand RNA, or polyinosinic acid-polycytidylic acid copolymer and also that the effect was not due to a direct toxic action of the endotoxin or double-strand RNA, which under the conditions used did not influence the growth of the target cells. Our experiment supports these results and may provide an explanation of 1 of the mechanisms of antitumor action.

PDP enhanced antibody response in mice bearing solid tumors. This result shows the adjuvant action of PDP from Days 5 to 15, while the other derivatives, including dextran, did not show this effect (Table 5; Chart 7). Thus, PDP has a host-mediated antitumor action against Ehrlich solid tumor.

Differences in humoral and cellular adjuvant activities of PDP with ascites tumors were not revealed, but statistical differences were shown against the tumor-bearing control groups (Tables 6 and 7; Chart 8). These results show that the combined antitumor action of PDP and mitomycin C is due to the stimulation of the immune mechanism by PDP.

During the course of the study of combination therapy, it was revealed that DP shows synergistic effect with mitomycin C. Thus, a phosphate group in both PDP and DP may...
Antitumor Activity of Dextran Derivatives

Chart 7. Effect of PDP on antibody formation in Ehrlich solid tumor-bearing mice. Mice were implanted s.c. with 1 × 10⁶ Ehrlich tumor cells into right groin; beginning 24 hr later, a solution of PDP (100 mg/kg/day) was injected i.p. for 10 successive days. Sheep erythrocytes were injected into the tail vein 4 days prior to determining the number of plaque-forming cells. O O, tumor control; x x, tumor + PDP (100 mg/kg/day); • •, normal control; x x, normal + PDP (100 mg/kg/day).

Table 5
Effect of dextran derivatives in vivo on the appearance of antibody-producing spleen cells in mice after the i.v. injection of sheep RBC

| Dextran and dextran derivative treatments, 100 mg/kg/day i.p. for 10 days. |
|---|---|---|---|---|
| Av. plaque-forming cells/spleen | Range | Ratio (%) | p |
| Tumor bearing* | | | | |
| Control | 13,799 | 9,058-18,540 | 116 | |
| Dextran | 11,123 | 8,306-13,222 | 93 | |
| PD | 12,489 | 8,273-15,798 | 105 | |
| DP | 16,534 | 14,940-17,940 | 139 | <0.05* |
| PDP | 33,376 | 27,278-40,180 | 280 | <0.01* |
| Normal | | | | |
| Control | 11,931 | 9,331-14,891 | 100 | |
| Dextran | 8,629 | 4,584-15,001 | 72 | |
| PD | 14,937 | 12,218-17,085 | 125 | |
| DP | 13,276 | 6,021-19,238 | 111 | |
| PDP | 14,009 | 13,190-15,529 | 117 | |

* dd mice were implanted with 1 × 10⁶ Ehrlich tumor cells s.c.

Table 6
Effect of dextran derivatives with mitomycin C in vivo on the appearance of antibody-producing spleen cells in mice after the i.v. injection of sheep RBC

| Dextran and dextran derivative treatments, 1 mg/kg/day i.p. for 5 days; mitomycin C treatments, 0.5 mg/kg/day i.p. for 5 days. |
|---|---|---|---|---|
| Av. plaque-forming cells/spleen | Range | Ratio (%) | p |
| Normal control | 8531 | 5892-9870 | 100 | |
| Tumor bearing* | | | | |
| Control | 2568 | 1483-3649 | 30 | |
| Dextran + mitomycin C | 2261 | 1151-4037 | 27 | |
| PD + mitomycin C | 2125 | 1876-2400 | 25 | |
| DP + mitomycin C | 2753 | 2358-3029 | 32 | |
| PDP + mitomycin C | 4176 | 3225-5689 | 49 | <0.05* |

* dd mice were implanted with 1 × 10⁶ Sarcoma 180 tumor cells i.p.

Table 7
Effect of PDP with mitomycin C on the induction of delayed-type hypersensitivity to bovine serum albumin in normal and Sarcoma 180 ascites tumor-bearing mice

| PDP treatments, 1 mg/kg/day i.p. for 5 days; mitomycin C treatments, 0.5 mg/kg/day i.p. for 5 days. |
|---|---|---|---|---|
| Increase in footpad thickness* (24-hr reading 0.1 mm) |
| Materials | Experiment 1 | Experiment 2 |
| Normal control | 1.9 ± 0.4* | 1.7 ± 0.3 | |
| Tumor-bearing control | 0.3 ± 0.2 | -0.3 ± 0.4 | |
| PDP | 0.2 ± 0.1 | 0.0 ± 0.1 | |
| Mitomycin C | 1.1 ± 0.5† | 0.4 ± 0.1 | |
| PDP + mitomycin C | 1.6 ± 0.2‡ | 1.0 ± 0.2‡ | |

* Ten mice/group.
† Mean ± S.E.
‡ p < 0.05, versus the tumor-bearing control group.
‡ p < 0.01, versus the tumor-bearing control group.

Chart 8. Effect of single and combination treatment with mitomycin C and PDP on antibody formation in Sarcoma 180 ascites tumor-bearing mice. Mice were implanted i.p. with Sarcoma 180 ascites tumor; beginning 24 hr later, a solution of PDP in 0.9% NaCl solution and/or mitomycin C was injected i.p. for 5 successive days. O O, normal control; □ □, tumor + PDP (1 mg/kg/day) + mitomycin C (0.5 mg/kg/day); O O, tumor + mitomycin C (0.5 mg/kg/day); □ □, tumor + PDP (1 mg/kg/day); O O, tumor control.
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play an important role in the tumor growth-inhibitory effect.

Niitani et al. (18, 19, 23) reported that the synergistic effect of dextran sulfate and mitomycin C depended upon the increased release of lysosomal enzymes. In preceding studies, we have investigated the interferon-including activity of DP (25, 28) and have shown that the properties of the induced interferon were identical with that evoked by bacterial endotoxin. The phosphate residue of the polysaccharide phosphate is undoubtedly essential for both antitumor and interferon-inducing activities, although no interpretation has been made on the relationship between the biological activities of these phosphorylated polysaccharides.

ACKNOWLEDGMENTS

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