Association of Macrophage Activation with Antitumor Activity by Synthetic and Biological Agents

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SUMMARY

Treatment of normal BALB/c mice i.p. with a number of adjuvants, including pyran copolymer, the copolymer of polynosinic and polycytidylic acids, Bacillus Calmette-Guérin, glucan, and dextran sulfate, rendered macrophages nonspecifically cytostatic for syngeneic tumor cells. Macrophage activation was highly dose dependent. The validity of the inhibition of DNA synthesis assay for measuring macrophage-induced cytostasis of target cells was proven by demonstrating a concurrent decrease in RNA synthesis and a reduction in viable tumor cell number. Moreover, conditioned supernatants from pyran-activated macrophages did not significantly decrease [3H]thymidine incorporation by freshly added leukemia cells. Biological or synthetic agents that activated macrophages were generally effective systemic antitumor agents against the M109 lung carcinoma. Drugs that did not activate macrophages, such as typhoid vaccine, tilorone, levamisole, WY-13876, and thymosin, were ineffective in prolonging the life of tumor-bearing mice. Pyran treatment i.p. was the most effective antitumor adjuvant in two separate tumor models, and suppression of tumor growth appeared to be related not only to an increase in macrophage tumoricidal function, but also to a larger influx of macrophages responding at the tumor site.

INTRODUCTION

A variety of biological and chemical adjuvants have the ability to enhance the metabolic and functional activity of mononuclear phagocytes. Although the mechanism of macrophage activation by these agents is quite dissimilar, the end result is the same; these cells display heightened tumoricidal and microbicidal activity (7, 10, 15, 20, 22), enhanced phagocytosis, and the ability to amplify the afferent limb of immune responses (12). Macrophages may either be directly activated by interferon inducers (e.g., lipid A, double-stranded RNA, and pyran copolymer) (1, 19) or may require activating factors from sensitized T-derived lymphocytes in the presence of appropriate antigen (2, 6, 17). We have presented indirect evidence that the ability of agents to render macrophages cytotoxic for tumor cells correlates with their capacity to enhance antitumor resistance (22).

Previous evidence showed that pyran copolymer markedly increased resistance of BALB/c mice bearing the M109 lung carcinoma (18, 21). Systemic treatment with pyran resulted in a prominent histiocytic infiltrate invading the tumor in which macrophages were often associated with necrotic tumor cells. Similarly, macrophages were recovered from pyran-treated animals, which potently arrested DNA synthesis of M109 tumor cells in vitro. The sensitivity of the M109 tumor to pyran copolymer and its lack of responsiveness to numerous cytoxic agents (25) suggested that this tumor might have the potential to identify alleged immunopotentiating antitumor agents. The present study compares pyran to other adjuvants in regard to both systemic antitumor activity and macrophage activation. The IDS2 assay for measuring tumor cytostasis was found to be a sensitive and reliable index of macrophage function. The presence of growth-inhibitory macrophages following adjuvant therapy correlated with significant increases in survival times of tumor-bearing mice.

MATERIALS AND METHODS

Mice. Male BALB/c and C57BL/6 mice, 6 to 8 weeks old, were obtained from the Mammalian Genetics and Animal Production Section, NIH, Bethesda, Md. All animals weighed at least 23 g before they were used for experimentation.

Drugs. The description and source of all adjuvants incorporated into this study are listed in Table I. All drugs were made up in Dulbecco's phosphate-buffered saline, adjusted to pH 7.2, and given i.p. at 1% body weight.

Target Cell Cultures. Cell strains derived from the M109 alveolar carcinoma and MBL-2 lymphoblastic leukemia have been established and maintained in RPMI-FCS. Both cell lines have been shown to be free of Mycoplasma contamination (Microbiological Associates, Bethesda, Md.).

Tumor Testing. The M109 lung carcinoma, which arose spontaneously in a BALB/c mouse in 1964, was received from Dr. Ruth I. Geran, Drug Research and Development, National Cancer Institute, NIH. The colon tumor 26, an undifferentiated colonic carcinoma originally induced in a BALB/c mouse by N-nitroso-N-methyl-urethan (3), was gen-

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The abbreviations used are: IDS, inhibition of DNA synthesis; RPMI-FCS, Roswell Park Memorial Institute Medium 1640 supplemented with 20% heat-inactivated (56° for 30 min) fetal calf serum, gentamicin (100 μg/ml), 0.075% NaHCO3, and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer; MST, median survival time; poly(I):poly(C), copolymer of polynosinic and polycytidylic acids; BCG, Bacillus Calmette-Guérin.
Macrophage Activation and Antitumor Activity

**Agents tested for their antitumor activity and ability to produce growth-inhibitory macrophages**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Source</th>
<th>Dose and route of administration (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levamisole</td>
<td>Janssen Pharmaceutical, Beerse, Belgium</td>
<td>10, i.p.</td>
</tr>
<tr>
<td>Poly(l)-poly(C)</td>
<td>Miles Laboratories, Inc., Elkhart, Ind.</td>
<td>10, i.p.</td>
</tr>
<tr>
<td>Pyran copolymer (NSC-46015)</td>
<td>Hercules Research Center, Wilmington, Del.</td>
<td>25, i.p.</td>
</tr>
<tr>
<td>Thiomusin (calf thymus extract Fraction 5)</td>
<td>Merrell-National Laboratories, Cincinnati, Ohio</td>
<td>50, i.p.</td>
</tr>
<tr>
<td>BCG, Pasteur strain</td>
<td>Dr. S. D. Chaparas, Bureau of Biology, Food and Drug Administration, Rockville, Md.</td>
<td>$10^7$ organisms/mouse, i.p.</td>
</tr>
<tr>
<td>Glucan</td>
<td>Dr. N. R. DiLuzio, Tulane University School of Medicine, New Orleans, La.</td>
<td>25, i.p.</td>
</tr>
<tr>
<td>Thymosin</td>
<td>Dr. A. L. Goldstein, University of Texas Medical Branch, Galveston, Texas</td>
<td>10, i.p.</td>
</tr>
<tr>
<td>Typhoid vaccine (U.S.P.)</td>
<td>Eli Lilly Company, Indianapolis, Ind.</td>
<td>$\sim 10^8$ killed organisms/mouse, i.p.</td>
</tr>
</tbody>
</table>

*Please note: Table 1 is not visible in the image.*

...serously supplied by Dr. F. Schabel from Southern Research Institute, Birmingham, Ala. Single cell suspensions were prepared from the tumors by enzymatic digestion of minced tissues with 0.25% trypsin. For adjuvant studies, $5 \times 10^6$ viable cell suspensions, suspended in serum-free Roswell Park Memorial Institute Medium 1640, were injected s.c. into the inguinal region of each BALB/c mouse. Selected adjuvants or placebo were administered i.p. on Day 7 after tumor inoculation. Deaths of mice were recorded daily, and MST's were calculated. The percentage increase in life span of adjuvant-treated groups in comparison to the normal control groups (C) was calculated by $(T/C - 1) \times 100$. The mean survival of the adjuvant-treated groups was 10% neutral formalin. These tissues were sectioned and routinely stained with hematoxylin and eosin.

**RESULTS**

Association of Antitumor Activity of Biological and Synthetic Agents with Macrophage Activation. The biological and synthetic adjuvants were tested for systemic antitumor activity against the syngeneic metastasizing M109 lung carcinoma. The MST of tumor-bearing mice that received placebo was 35.5 days. Pyran copolymer at $25 \text{ mg/kg}$ on Day 7 after tumor inoculation was the most significant ($p < 0.001$) at prolonging survival times (MST = 55 days) of tumor-bearing mice (Chart 1a). Poly(l)-poly(C), BCG, and glucan

*Please note: Table 1 is not visible in the image.*
also exhibited significant antitumor activity (MST of 44.0, 42.5, and 42.0 days, respectively), whereas dextran sulfate, typhoid vaccine, tilorone, levamisole, WY-13876, and thymosin were without effect under the experimental conditions used.

These agents were tested under similar conditions for the ability to produce growth-inhibitory macrophages. Peritoneal macrophages were collected 6 days after i.p. administration of drugs to normal, non-tumor-bearing mice and tested against syngeneic M109 target cells (Chart 1b). All polyanions [pyran, poly(l)-poly(C), and dextran sulfate] markedly stimulated BALB/c macrophages to inhibit M109 DNA synthesis ($p < 0.001$). In addition, the biological agents BCG and glucan rendered macrophages cytostatic, whereas typhoid vaccine, tilorone, levamisole, WY-13876, and thymosin were without significant effect. These agents, which failed to activate macrophages, were similarly ineffective in prolonging the life span of tumor-bearing mice. Biological or synthetic agents that activated macrophages, such as pyran, poly(l)-poly(C), BCG, and glucan, were generally effective systemic antitumor agents against the M109 lung carcinoma.

To determine whether a positive correlation existed between macrophage activation and antitumor activity in a different tumor system, the various agents were tested against the colon tumor 26. Only pyran copolymer was found to significantly ($p < 0.01$) enhance BALB/c resistance in 2 separate screens. The MST's were increased 28.2 and 46.0%, respectively, by pyran treatment.

Since pyran was the only effective adjuvant against the colon tumor 26, the histopathology of the host response against the primary tumor was studied. Histologically, the colon tumor 26 represents a poorly differentiated carcinoma interspersed with spindle cell-like areas (Fig. 1). As early as 2 days after pyran therapy (Day 9), the host tissue showed signs of cellular activity with increasing numbers of histiocytes, lymphocytes, and some granulocytes at later intervals (Days 11 and 13). At Day 18, histiocytes and macrophages predominated around and within the tumor tissue. The tumor was clearly demarcated from the surrounding fibrofatty tissue which showed a heavy infiltration of histiocytes, numerous macrophages with foamy appearance of the cytoplasm, as well as some lymphocytes (Fig. 2). These cells were observed infiltrating tumor tissue in groups from the periphery, encircling viable-looking tumor cells, and separating the tumor tissue toward the center. Although the migration of granulation tissue due to pyran therapy was less developed than previously reported in the M109 lung tumor (21), the attack of histiocytes and macrophages against the colon carcinoma cells was more obvious. In contrast, the host reaction in Dulbecco’s phosphate-buffered saline-treated animals bearing the colon tumor 26 was nearly absent. The growth was more progressive with infiltration of the tumor up to the deeper layers of the skin (Fig. 3). Tumor cell necrosis was only moderate in comparison to the tumors of pyran-treated animals taken at similar intervals.

**Dose Response of Macrophage Activation In Vivo.** Further experiments were performed to test the dose dependency of adjuvant-induced macrophage activation. Drugs were administered i.p. at doses ranging from 100 to 0.1 mg/kg. Macrophages were harvested on Day 6. Activation by polyanions was sharply dose dependent (Chart 2). Pyran copolymer and poly(l)-poly(C) treatment produced optimal macrophage stimulation at 10 mg/kg, whereas dextran sulfate required 25 mg/kg. Antitumor activity by pyran is similarly dose dependent, although a single i.p. treatment of pyran on Day 7 after M109 tumor implantation is effective over a dose range of 1 to 100 mg/kg (18). Macrophage activation by glucan was active over a broader range from 1 to 25 mg/kg. Higher levels of drug were not active.

**Validity of IDS Assay.** Since macrophages have been reported to secrete thymidine or an analog thereof (24), we tested whether our IDS assay actually reflected target cell proliferation or merely competition of macrophage secretions for radioactive thymidine used for pulsing the cells. MBL-2 lymphoblastic leukemia cells were used as targets. Due to their nonadherent nature in cell culture, these cells have the advantage of readily being distinguished from macrophage effectors and are sensitive to the cytotoxic effects of activated macrophages (19). Macrophages acti-
Macrophage Activation and Antitumor Activity

The vital role that mononuclear phagocytes play in control of tumor growth and its dissemination are now being justly recognized. The conversion of the resting macrophage to an activated state by polyanions or soluble mediators constitutes a primitive yet highly effective surveillance and potent antitumor mechanism. Once activated, macrophages can regulate cell proliferation (10) and selectively destroy cells with abnormal growth properties (9).

The studies presented here show that the ability of biological and synthetic agents to render macrophages cytostatic for tumor cells correlated with their capacity to increase life span of mice bearing the syngeneic M109 lung carcinoma. Macrophage activation by pyran, poly(I)-poly(C), glucan, and dextran sulfate was sharply dose dependent, with supraplimal levels of drug having lost their activity. The reason for this dose dependency is unknown, although direct macrophage activation by polyanions in vitro shows similar kinetics (19). Pyran was the most effective adjuvant in regard to both macrophage activation and antitumor activity in 2 tumor models. Since pyran is not directly toxic for tumor cells in vitro, modulation of macrophage activity has been implicated in the antitumor activity (21). Pyran has previously been shown to suppress the growth of numerous solid tumors (15, 18, 21, 23, 25) and is capable of producing a significant number of “cures” when combined with remission-inducing chemotherapy against the Lewis lung carcinoma and LSTRA murine leukemia (13). The antitumor activity of pyran has been studied in depth against the primary M109 lung carcinoma and its metastases (18, 21). Systemic pyran therapy was active over a wide range of doses from 1 to 100 mg/kg/day, and multiple doses were not significantly better than single treatments. A single treatment was effective even relatively late in the course of neoplasia (Day 14) in a system where the MST of control mice is about 30 days.

Pyran copolymer was previously found to be toxic in Phase 1 clinical trials involving advanced cancer patients (16); toxic side effects, including thrombocytopenia and hypotension, precluded further study. However, at that time, the ability of pyran to enhance host survival against neoplasia was believed to result from direct antimitotic effects. Of particular significance are our findings that pyran is effective over a large range of doses and that multiple treatments do not give additional protective effects (21). Moreover, we present evidence that pyran dosage at high levels inhibits macrophage function. It now appears that pyran therapy can be tailored to the individual tumor system to minimize patient toxicity and retain good activity.

Although it is possible that pyran and the biological adjuvants stimulate other cell types that both inhibit tumor growth and, in turn, activate macrophages, in vivo studies have further implicated activated macrophages as effectors of tumor resistance. Histopathological studies have revealed an intense histiocytic reaction in the connective tissue surrounding the s.c. primary tumor in mice treated intrasplenially with BCG (8) or glucan (11). Intimate contact between macrophage and target cell was a requirement for tumor cell destruction. The presence of large numbers of histiocytes in several untreated animal tumors was similarly associated with tumor rejection and the lessened likelihood of metastasis (4). Thus, the major limitation of the macrophage effector arm of the immune response appears to be cell concentration at the primary or disseminated tumor site. It is tempting to compare pyran copolymer with BCG, since pyran copolymer therapy provoked histiocytosis around the s.c.-transplanted M109 lung carcinoma (21) and colonic carcinoma 26 in BALB/c mice, and since both agents activate macrophages in vivo. However, 3 distinct differences are involved: (a) there is no granuloma development as would be seen with BCG; (b) macrophage activation and mobilization by BCG requires functional T-cells (17), whereas activation by pyran is direct (19) and does not require an immunocompetent host; and (c) BCG requires intrasplenic therapy to produce a migration of histiocytes to the tumor (8), whereas i.p. pyran treatment produces a histiocytic reaction at the s.c. tumor site (21, 23).

Pyran therapy also reduces the number of pulmonary lesions that develop after the i.v. inoculation of M109 tumor cells (18). Snodgrass et al. (23) have shown that small numbers of macrophages, presumably of hematogenous origin, accumulated in the pulmonary interstitium of pyrantreated animals. There were regions where macrophage accumulations formed nodules that disrupted much of the normal architecture. It is tempting to speculate that these pyran-activated macrophages have a surveillance function.
in inhibiting or controlling metastatic cell growth. Similarly, Fidler (5) has reported that activated macrophages injected i.v. inhibit pulmonary metastasis of B-16 melanoma cells.

The data presented are consistent with the hypothesis that nonspecifically activated macrophages are major effectors of the antitumor resistance induced by both synthetic and biological immunopotentiators. The effect of pyran on modulating host immunological factors, even in an advanced tumor system (21), its ability to augment specific immune responses (14), its compatibility with cytoreductive chemotherapy (13), and its effect on inhibiting distant metastasis (18) strongly support the potential use of pyran copolymer as an adjuvant to conventional tumor treatment modalities.

ACKNOWLEDGMENTS

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REFERENCES

Fig. 1. Colon tumor 26. Poorly differentiated carcinoma with intertwining arrangement of tumor cells. Mitotic figures are abundant. × 250.

Fig. 2. Numerous macrophages (short arrows) with typical foamy cytoplasm together with some clusters of lymphocytes (long arrow) invading tumor tissue, 18 days after pyran treatment. × 250.

Fig. 3. Tumor surrounded by connective tissue without host reaction. Expansion of the periphery of the tumor up to the deeper layers of the skin (hair follicle and sebaceous gland in right upper corner), 18 days after placebo treatment. × 250.
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