Response of Tumors to Biologic Modifiers and Chemotherapeutic Agents

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The use of the FANFT model has facilitated clinical trials which are now beginning to have an impact on the therapy of human bladder tumors. We studied C. parvum and Glucan immunotherapy of FANFT tumors, and demonstrated the enhancement of tumor growth following Glucan administration. Weekly injections of cyclophosphamide (CY) retarded tumor growth and increased survival. Combining C. parvum or Glucan with CY resulted in greater tumor growth inhibition than CY alone. Our data support the concept that the therapeutic effectiveness of CY can be improved by biologic modifiers that alter its rate of activation.

(Key Word: C. parvum, Glucan, FANFT tumor, Immunotherapy, Chemoimmunotherapy)

INTRODUCTION

The role of immunotherapy in the management of tumors has recently emerged as a new discipline. Its rapid development as a major approach to cancer treatment was stimulated by demonstrations that immuno-enhancing agents effected tumor regression in animal systems. The agents most frequently used for active non-specific immunotherapy are Bacillus Calmette-Guerin (BCG) and the anaerobic diphtheroid bacterium, Corynebacterium parvum (C. parvum), in the United States (17, 18). The latter has been found to be the most potent bacterial stimulant of the macrophage system currently available. C. parvum has also been shown, in experimental tumors, to be an effective immunoadjuvant and to cause tumor regression when combined with cytotoxic agents (15).

Glucan, a β 1-3-polyglucosidic component of the cell wall of Saccharomyces cerevisiae, has been demonstrated by DiLuzio et al (10, 11) to be a novel reticuloendothelial stimulant that enhances cellular and humoral immunity. The extent to which the substance inhibits growth of established growing tumors has not been extensively studied.

The efficacy of immunostimulants depends on the type of tumor being treated (25) as well as the mode and timing of the treatment (4). It has been found that, under certain conditions, BCG and C. parvum displayed no beneficial effects, and in some cases they even led to the promotion of tumor growth (2, 21). Also, in recent years, attempts have been made to increase the effectiveness of chemotherapeutic agents and enhance tolerance to
chemotherapy through the administration of non-specific immunostimulants. The purpose of the present study was to determine the activity of certain immunotherapeutic agents against carcinogen N(4- (5-nitro-2-furyl)-2-thiazolyl) formamide (FANFT)-induced murine bladder tumors and assess their anti-tumor effect when combined with a cytotoxic agent (cyclophosphamide).

MATERIALS AND METHODS

ANIMALS: Six and eight-week-old inbred female C3H/He mice were used (obtained from NIH, NIC Mammalian Genetics, and Animal Product Section, Frederich Cancer Research Center, Frederich, MD, 21701). The mice were fed a diet of laboratory purina chow containing 0.1 percent of FANFT to induce a tumor in the bladder. Tumors were then propagated by transplantation into the legs of syngeneic mice.

PREPARATION OF TUMOR CELLS: Single-cell suspensions of tumor were prepared by a modification of the method of Madden and Burk, as previously described (6). Briefly, tumor tissue was aseptically excised, finely minced with scissors, and placed in a flask in which enzymatic dissociation was carried out in Hank's balanced salt solution (HBSS) containing 0.25% trypsin. The mixture was agitated on a magnetic stirrer for 20 minutes at 37°C and then filtered through a fine wire mesh. The resulting cell suspension was centrifuged at 1,500 rpm for 10 minutes at 20°C, resuspended, and washed several times with HBSS. The cells were again resuspended and the number of viable cells was determined in a hemocytometer by counting those that excluded 0.05% trypan blue. The final concentration of viable cells was adjusted to $1 \times 10^5 \sim 1 \times 10^7$ per 0.1 milliliter.

EXPERIMENTAL PROCEDURES: C. parvum was obtained from Wellcome Research Laboratories (Research Triangle, North Carolina). It was also freshly prepared and administered weekly intraperitoneally or subcutaneously. Cyclophosphamide (cytoxan) was also freshly prepared and administered weekly intraperitoneally. Each animal received $1 \times 10^5$ cells per 0.1 ml. After tumor inoculation, animals were randomized into control groups of 20 mice and treatment groups with a minimum of 20 mice per group. Limbs were palpated biweekly, and palpable tumors were measured with Vernier calipers. In these experiments, therapy was begun when tumors measured 6-7 mm in diameter. The optimal dose determined by previous experiments was 0.05 mg/gm body weight or about 1 mg per animal. Groups of mice were injected with isotonic saline (0.1 ml/mouse). C. Parvum alone (1.0 mg/mouse), cyclophosphamide alone (75 mg/kg), or C. parvum (1.0 mg/mouse) plus cyclophosphamide (75 mg/kg). Animals were treated until 50% of the control groups were dead.

Glucan was kindly provided by Dr. DiLuzio, Department of Physiology, Tulane University School of Medicine, New Orleans, Louisiana. Dilutions were made in sterile 5% dextrose solution. Glucan was injected intraperitoneally twice weekly in two different doses.

One experiment was designed to determine the dose-response relation-
ship and the temporal relationship of Glucan to tumor inoculation. Groups of mice were injected with 5% dextrose solution (0.5ml/mouse), Glucan (0.9 mg/mouse) three weeks before tumor inoculation, Glucan (0.9 mg/mouse) plus tumor cells on the same day, Glucan (0.9 mg/mouse) five days after tumor inoculation, Glucan (0.45 mg/mouse) when the tumor measured 5-6 mm in diameter, and Glucan (0.45 mg/mouse) plus tumor cells on the same day.

A second experiment was designed to determine if the effects of chemotherapy on established FANFT tumors were enhanced by immunotherapy with Glucan. The experimental procedure was the same as that for the C. parvum experiment. The dosage used in this study was 0.45 mg per mouse.

**STATISTICS:** the data presented in these studies were statistically evaluated using the Student \(t\)-test and \(Z\)-test to determine the significance of proportions. A value of \(p<0.05\) was considered significant.

**RESULTS**

**FANFT TUMOR GROWTH CURVE AND IMMUNOCENICITY:** The tumor growth pattern was predictable after subcutaneous injection in the lower leg of the C3H/He mice. We have recently established the immunogenicity of this tumor in classical tumor challenge experiments, the results of which are depicted in Figure 1. Animals bearing 6 and 8 mm tumors rejected subsequent tumor challenges after amputation of the tumor bearing limb. All animals inoculated with \(10^6\) cells developed tumors with an average diameter of 14 mm by day 30. Only 50% of previously immunized animals (tumors amputated when 6 and 8 mm in diameter) developed tumors, with an average diameter of 7 mm on day 30. Four of 14 amputated host animals challenged with \(10^5\) cells developed small tumors. When the tumor size was allowed to become greater than 13 mm before amputation of the limb, all animals accepted subsequent tumor challenge with growth of challenge tumors paralleling that of controls. Implantation and growth of different murine tumors were not affected by immunization with the FANFT tumor.

![Fig. 1 FANFT tumor growth curve and immunogenicity.](image-url)
C. PARVUM IMMUNOTHERAPY AND CONCOMITANT CHEMOTHERAPY: C. parvum was found to be equally effective when administered either subcutaneously or intraperitoneally. In previous experiments (7), the agent was found to be effective when administered at the time of tumor inoculation. In these experiments, therapy was begun when the tumor measured approximately 6mm in diameter.

By the second week of therapy, tumor growth was significantly inhibited \((p<0.05)\) in the C. parvum group when compared with the controls. Cyclophosphamide alone had a growth inhibition effect almost identical to that of C. parvum alone. When cyclophosphamide was combined with C. parvum and administered subcutaneously, growth inhibition was greater than that achieved by either agent used alone \((p<0.05)\). The most significant effect was achieved by a combination of C. parvum given intraperitoneally plus cyclophosphamide \((p<0.01)\) (Fig. 2). In this group, three out of 20 animals showed complete regression of the tumor which was sustained throughout subsequent follow-up until death.

Survival of treated animals is depicted in Figure 3. Survival of all treated groups was greater than that of the control group. Survival at the point which 50% of control animals were dead was significantly greatest in the group that received C. parvum subcutaneously plus cyclophosphamide. Although the survival curves became less disparate after continued long-term follow-up, this group still maintained a greater survival rate than the cyclophosphamide alone group.

Survival of animals that received C. parvum alone either subcutaneously or intraperitoneally was almost identical to that of the group receiving C. parvum subcutaneously plus cyclophosphamide, and this is not depicted in Figure 3. The group that showed the greatest inhibition of tumor growth (C. parvum intraperitoneally plus cyclophosphamide) did not show a greater survival rate than the cyclophosphamide group alone because of apparent toxicity resulting from this method of administration.

![Graph](Fig. 2) Effect of C. parvum and cyclophosphamide on the established measurable tumors in C3H mice.
GLUCAN IMMUNOTHERAPY AND IMMUNOPROPHYLAXIS: Tumor growth was not reduced in any group treated with Glucan (Table 1). Glucan administered three weeks before tumor inoculation produced no effect on the incidence of tumor takes. On the other hand, there was a significant increase in the average tumor diameter in animals injected with Glucan (0.90 mg) three weeks before tumor inoculation and with Glucan (0.45 mg) plus tumor cells (p<0.005 compared with controls). By day 54, most animals died of large local tumor growth.

Table 1 Effect of Glucan immunotherapy on modification of tumor growth

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean tumor diameter (mm)* on Day 39</th>
<th>P value</th>
<th>Number in group</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% dextrose</td>
<td>13.5 ± 4.0</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>Glucan, 0.90 mg 3 wks before tumor inoculation</td>
<td>19.8 ± 1.6</td>
<td>p&lt;0.05**</td>
<td>5</td>
</tr>
<tr>
<td>Glucan, 0.90 mg plus tumor cells</td>
<td>16.8 ± 9.2</td>
<td>N.S</td>
<td>5</td>
</tr>
<tr>
<td>Glucan, 0.90 mg 5 days after tumor inoculation</td>
<td>15.3 ± 2.5</td>
<td>N.S</td>
<td>6</td>
</tr>
<tr>
<td>Glucan, 0.90 mg when tumor measured 5-6 mm</td>
<td>18.8 ± 1.3</td>
<td>N.S</td>
<td>7</td>
</tr>
<tr>
<td>Glucan, 0.45 mg plus tumor cells</td>
<td>20.2 ± 3.4</td>
<td>p&lt;0.05**</td>
<td>7</td>
</tr>
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*mean ± SD
**Significant increase in average tumor diameter when compared to controls
GLUCAN PLUS CYCLOPHOSPHAMIDE TREATMENT: Weekly intraperitoneal injections of cyclophosphamide, 74mg/kg, retarded tumor growth (p<0.05) and increased survival (Figs. 4 and 5). Combining Glucan with cyclophosphamide resulted in greater tumor growth inhibition than cyclophosphamide alone, although the difference in mean tumor diameter on day 49 was not statistically significant. Glucan alone had no effect on tumor growth. The survival of tumor-bearing mice that received Glucan plus cyclophosphamide was greater than either that of the control mice or those treated with Glucan only (p<0.05) although there was a major difference in the survival curves of control animals in figs. 3 and 5.

Fig. 4 Effect of Glucan and cyclophosphamide on established measurable tumors in C3H mice.
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DISCUSSION

The FANFT murine bladder tumor appears in 80% of C3H/He mice after nine months of daily feeding of a diet containing 0.1% of the carcinogen. These tumors are transitional carcinomas which are papillary and invasive, and resemble human bladder cancer grossly and microscopically (6). They usually kill by local extension, but occasionally metastasize to the lungs and peritoneum. Tumors have also been induced in dogs and Fisher rats. Several murine lines were established in our laboratories recently and have been maintained by subcutaneous implantation. We have used this model for chemotherapeutic trials and shown that the tumor is sensitive to cyclophosphamide (Cytoxan) alone. Combinations of Cytoxan with Cis-platinum, or Cis-platinum alone, were also effective in inhibiting tumor growth and prolonging survival. The tumors have also been shown to be somewhat responsive to Adriamycin. Therefore, the responsiveness of the tumor to cytotoxic drugs closely parallels recent clinical experience (8, 29).

It is possible that the FANFT tumor may play a role in assessing immunotherapeutic agents. The studies of deKernion et al (7) have permitted a tumoricidal evaluation of potentially useful immunotherapeutic agents in the treatment of bladder tumors. These authors (9) also reported that C. parvum was more effective when administered in conjunction with cyclophosphamide, there was greater inhibition of tumor growth and increased survival than when either agent was used alone. We have recently established the immunogenicity of the tumor in classical tumor challenge experiments. Our results support the study of Javadpour et al (19) who found that FANFT tumor cells were immunogenic with no cross-reactivity with syngeneic fetal...
Morales et al (27) reported that immunization with irradiated FANFT tumor cells (MBT-2) or hypotonic membrane preparations from this tumor protected the animals against challenge with viable MBT-2 cells.

The present study demonstrated significant anti-tumor activity of cyclophosphamide in the FANFT tumor model. It was if even greater interest that the combination of systemic immunostimulation using C. parvum and cyclophosphamide as cytoreductive chemotherapeutic agents produced dramatic growth inhibition of tumors. Several authors (14,15,16) have demonstrated a synergism between C. parvum and cyclophosphamide when used together. Amiel and Berardet (1) provided an experimental model of active immunotherapy preceded by chemotherapy. Immunotherapy given after a course of chemotherapy was required to reduce the number of leukemic cells. This model allows for the action of immunotherapeutic agents tested on the residual population, i.e., cells that lodge in sites least accessible to chemotherapy.

In addition to bacterial adjuvants, DiLuzio and his associates(10,11) have demonstrated that Glucan, a β1, 3-glucosidic polyglucose derived from the cell wall of Saccharomyces cerevisiae, is a potent immunopotentiator. The administration of Glucan to mice or rats is associated with the induction of a hyperphagocytic state resulting in the hypertrophy of major reticuloendothelial organs which results from an increase in both the size and number of macrophages. The neutral polyglucose is effective in enhancing both humoral and cellular immune responses to unrelated antigens (33). In addition, Glucan is non-antigenic and non-virulent as opposed to bacterial immunostimulants and therefore, may possibly induce fewer toxic complications (26).

Our studies were designed to evaluate the influence of Glucan on the FANFT tumor. Although no immunotherapeutic effects were shown and tumor growth was not reduced (in fact, there was an increase in the average tumor mass in Glucan treated animals), encouraging results were demonstrated when Glucan was used in conjunction with cyclophosphamide. When the tumor became palpable, the combination of Glucan and cyclophosphamide was effective in inhibiting tumor and prolonging survival to a greater extent than when cyclophosphamide was used alone. Recent studies by DiLuzio et al (12) demonstrated that concomitant chemotherapy with cyclophosphamide and immunostimulation with Glucan was effective in inhibiting metastases and prolonging the survival of rats with acute myelogenous leukemia.

Of even more interest is the finding that immunization with Glucan failed to induce protection against FANFT tumor development. The enhancement of tumor growth following BCG immunostimulation has been thoroughly demonstrated in some experimental models. Repeated administration of BCG has also been reported to depress cellular immunity in animals as measured by the delayed cutaneous hypersensitivity reaction to certain common skin antigens (22). Soloway (30) showed no decreased induction of experimental FANFT bladder tumors after chronic administration of BCG. We have recently shown that there is tumor enhancement following BCG immunostimulation (20).
It has also been observed that C. parvum can promote the growth of chemical-induced tumors (2). It is therefore possible that, under some circumstances, this agent might depress manifestations of cell-mediated immunity, thus leading to the promotion of tumor growth, even though systemic administration of C. parvum inhibited tumor growth in our studies. On the other hand, there was an increase in the average tumor mass in Glucan-treated animals. The responses to these opposite inhibiting and stimulating effects are presently unknown. Several investigators (2, 3, 23, 32) have made proposals for the mechanism(s) of tumor enhancement following non-specific immunostimulation as follows: (a) antigenic competition can exist between non-specific immunostimulants and tumors, (b) the induction of serum-blocking factors may occur in animals immunized with immunostimulants which is caused by the trapping of anti-tumor effector cells at the site of immunostimulants deposition, and (c) high doses of immunopotentiating agents may generate suppressor T cells.

It has been previously postulated that any immunopotentiating agent that can vary the immune response of a tumor-bearing host over an appreciable range should be capable of both stimulating and inhibiting tumor growth.

Activation of cyclophosphamide in vitro has been shown to be catalyzed by the same hepatic microsomal mixed-function oxidase system that is functional in oxidative metabolism of many other drugs (28). Recent results reported Farquhar et al (13) suggest that C. parvum and BCG inhibits the function of hepatic microsomal enzymes, and that this inhibition might be a common effect of bacterial adjuvants. However, after studying the action of C. parvum, Fisher et al (16) suggested that its action might not result entirely from its effect on metabolism of cyclophosphamide.

Their finding provided evidence indicating that the tumor inhibitor effect of C. parvum when combined with the chemotherapeutic agent cyclophosphamide, might be, at least partly, due to a mechanism other than its role as immunopotentiator. Even though metabolite protection was reduced, it is still possible that active compound(s) was preferentially produced. They also suggested that, contrary to the conclusions of others, at least in this system the therapeuric effectiveness of cyclophosphamide could be improved by agents that altered its role in activation. Their findings, together with the data from our studies, provide a rationale for investigation of such combined therapy in human malignancies.

Non-specific immunostimulants modified the increased susceptibility of chemotherapeutic agents and enhanced both the response and tolerance to chemotherapy. Our studies support the hope that chemoimmunotherapy is ideal for treatment of invasive bladder cancer in human beings.

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