ABSTRACT

The effect of treatment with the thymic factor thymostimulin (TP-1) on the survival rate of tumor-bearing mice was studied, using C57BL/6 mice inoculated with $1 \times 10^5$ Lewis lung carcinoma (3LL) cells. TP-1 given from inoculation day (4 mg/kg, twice weekly) caused a delay in the appearance of primary tumor [14.4 ± 1.1 (S.E.) days in control; 18.5 ± 1.4 days in TP-1-treated animals; $p < 0.05$], without changing ultimate survival rate. When primary tumor was resected, the incidence of fatal lung metastasis increased as a function of tumor size on resection day. TP-1 given after resection (same dose schedule) significantly increased survival rate as compared to resection only, provided that resected tumor diameter was <1.7 mm. The combination of TP-1 and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU; single i.p. injection, 50 mg/kg) was effective in either resected or nonresected primary tumor. Without resection, TP-1 with CCNU cured (more than 6 months free of tumor; untreated animals died within 30 to 44 days) 55% of the animals, as compared to 23% cured by CCNU alone ($p < 0.01$). With resection animal cure rates were: resection (resected tumor diameter, 0.7 to 1.7 mm) alone, 42% cured; resection with CCNU, 47% cured; resection with TP-1, 70% cured; resection with CCNU and TP-1, 100% cured (last two groups significantly different from resection only). The results indicate a profound effect of TP-1 in prolonging life and increasing cure rate of tumor-bearing mice. This effect was manifested when tumor load was small and was apparently more pronounced on metastatic than on primary tumor.

INTRODUCTION

The involvement of the immune system in the destruction of tumor cells has been a matter of active research ever since this idea was raised by Paul Ehrlich in 1908 (24). Amazingly, Ehrlich had foreseen the key importance of cell-mediated immunity in host defense against cancer, a prediction which was confirmed and established in the last 2 decades (13). Because a normal functioning thymus gland is necessary for the development and regulation of cell-mediated immunity (2, 26), the thymus and the hormones secreted from it should be considered in the analysis of tumor immunity and, ultimately, in our trials for immunological intervention against cancer. However, although the basic idea seems to be valid, the actual experimental data are still conflicting in this regard. The lack of thymus, either surgically removed or congenitally absent, was not consistently correlated with increased incidence of cancer (35). There are several reports indicating facilitation of tumor development in the absence of the thymus (1, 12, 16, 17, 36), some others with apparently opposed effect (16, 17, 21), and many studies that were not able to demonstrate a difference in tumor incidence between thymus-bearing and thymusless mice, even during the entire life span of the animal (1, 16, 17, 28, 29).

Much less is known about the effect of humoral factors isolated from the thymus on tumor development. Generally, the approach was to use immunodeficient or thymectomized, tumor-inoculated mice and to give thymic factors as a replacement or restorative treatment. No substantial effect on survival was observed in such experiments (9, 22) in spite of the documented effect of thymic factors in restoring some cell-mediated immune functions in neonatal thymectomized mice (2). Earlier reports with undefined thymus homogenate (19) or supernatant of such homogenate (20) indicated a protective effect against 20-methylcholanthrene-induced skin cancer in normal mice. Thymosin Fraction 5 when used in combination with chemotherapy was effective in prolonging the life of mice inoculated with Moloney leukemia cells (6). In another experimental approach, lymphocytes sensitized in vitro against fibrosarcoma cells had stronger antitumoral activity after in vitro incubation with thymic humoral factor (34). Thus, although some reports suggest a potential value of thymic factors in augmenting host immune reactivity against cancer, this antitumoral effect was not clearly demonstrated as yet.

The present study was undertaken in order to find out whether one can define conditions of thymic hormonal treatment which will have a real impact on survival of tumor-bearing animals. The design of the experiments was influenced by our previous studies (30) which indicate that thymic hormonal effect should be considered not only as correcting immunodeficiency states but also as regulating normal and mature immune reactivity. Consequently, we used healthy, mature, thymus-bearing mice rather than thymusless ones. The tumor selected for this study is 3LL, syngeneic in C57BL/6 mice and metastasizing to lungs. The thymic factor tested in these experiments was TP-1 (thymostimulin), a partially purified thymic extract of calf thymic extract of thymic humoral factor (34). This thymic hormone preparation was shown to increase the percentage of Thy-1-positive cells of mouse spleen and their responsiveness to phytohemagglutinin and concanavalin A stimulation but not to lipopolysaccharide (7). TP-1 stimulated the capacity of allogeneic mouse marrow cells to induce graft-versus-host response in irradiated mice (7). In humans, TP-1 was shown to regulate

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4 The abbreviations used are: 3LL, Lewis lung carcinoma; TP-1, thymic extract of Serono, also called thymostimulin; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (lomustine); PBS, phosphate-buffered saline.

MATERIALS AND METHODS

Animals. Six- to 10-week-old inbred male C57BL/6 mice were purchased from the Animal Breeding Center, The Weizmann Institute of Science (Rehovot, Israel). Mice weighed an average of 25 g at the start of the experiments.

Tumor Cells and Tumor Inoculation. 3LL is a tumor that arose spontaneously in a C57BL/6 mouse and has been maintained by serial passages in the same strain (11). Single-cell suspensions of 3LL tumors were prepared as follows. Small pieces of nonnecrotic tumor tissue were stirred at 37°C in PBS containing trypsin (100 μg/ml), DNase (25 μg/ml), and collagenase (100 μg/ml). All of these enzymes were purchased from Sigma Chemical Co., St. Louis, Mo. Cells were collected every 10 min and washed 3 times in PBS. Cell viability was determined by the trypan blue exclusion method. Only cell preparations with 95 to 100% viable cells were used for inoculation. One × 10^6 cells in 50 μl PBS were inoculated into the left hind footpad of the mice, using Hamilton’s syringe with repeated dispenser.

Follow-up of Tumor-Bearing Mice. Mice were randomized into groups of 8 to 15 animals/cage after tumor cell inoculation and individually labeled by ear marks. Primary tumor appeared as a visible small nodule at the site of inoculation within 7 to 15 days. Subsequent tumor growth was followed by determining tumor diameter every 2 to 3 days according to the following formula:

Tumor diameter = diameter of involved footpad - diameter of uninvolved footpad

Measurements were taken at the level of tumor inoculum and perpendicular to it, using a vernier caliper (Mitutoyo, Japan). Repeated measurements of 2 uninoculated hind footpads of the same mouse gave identical results. Mice were not sacrificed but were kept until death or, if tumor free, for 6 months at least. Untreated tumor-bearing mice died within 30 to 44 days after tumor cell inoculation.

Resection of Primary Tumor. Primary tumor was resected by amputating the tumor-bearing leg. Mice were anesthetized by ether, and amputation at midhigh level was performed using an electrosurgical knife (Leibel Farhiehm Co.). The animals passed this procedure extremely well.

Drugs and Drug Therapy. TP-1 (thymostimulin) was prepared by Bergesi and Falcetti (4) from the Istituto Farmacologico Serono, Rome, Italy, according to the following procedure. Calf thymuses were minced and extracted with 0.15 M dimethyl sulfoxide and injected i.p. into the mice, either daily (6 days/week) or every 3 to 4 days (twice per week). CCNU was dissolved in 50% dimethyl sulfoxide and injected i.p. in 0.1 ml. Dimethyl sulfoxide alone had no effect on animal survival or tumor growth.

Statistical Analysis. Survival data were analyzed using life tables and logrank test (27). The logrank significance level was estimated by comparing actual experimental distributions [calculated as (O - E)^2/E where O and E represent deaths observed and extent of exposure to risk of death, respectively] with those of appropriate χ^2 distributions (27).

RESULTS

Effect of TP-1 as Single Agent in Treatment of 3LL Tumors. Initial experiments were performed in order to determine the effect of TP-1 on tumor development. Mice inoculated with 1 × 10^6 3LL cells in the hind footpad were treated by different dose schedules of TP-1, starting on the day of tumor inoculation. In untreated mice, the mean interval time from inoculation of tumor cells to the appearance of primary tumor in the footpad was 14.3 ± 1.1 (S.E.) days. TP-1 treatment caused a delay of a few days in appearance of tumor. However, not every dose was equally effective (Chart 1). The best and statistically most significant effect was observed with TP-1 (40 or 4 mg/kg twice a week) or TP-1 (4 and 0.4 mg/kg 6 times a week). It was interesting that an optimum rather than a plateau curve of TP-1 was observed; i.e., both high- and low-dose schedules (40 mg 6 times a week and 0.4 mg twice a week, respectively) were not significantly different from control. On the basis of these results, we selected one dose schedule for further experiments (4 mg/kg twice a week).

Once a tumor became measurable, 2 stages could be observed in its growth, slow growth in the first few days, followed by a fast growth period. Tumor growth curves of treated and untreated mice were similar during the fast growth period. However, the period of slow growth was apparently longer in TP-1-treated mice (Chart 2), and these mice survived a few days more (Chart 3). These differences, however, were not statistically significant.

Chart 1. Titration of TP-1 effect on time of tumor appearance. C57BL/6 mice were inoculated with 1 × 10^6 3LL cells to footpad and TP-1 treatment with the indicated doses was started 1 day after tumor inoculation, 6 times or twice a week. * p < 0.05 (n = 14 for each point).
A treatment (4 mg/kg twice weekly) was started 1 day after tumor inoculation. Similar results were obtained in 2 subsequent experiments with a total of 35 single I.p. injection of CCNU (50 mg/kg) was given 9 days after tumor inoculation. A significantly higher survival rate of mice treated by CCNU alone (p < 0.01).

The cured rate of mice treated by CCNU plus TP-1 was significantly higher than was the survival rate of mice treated by CCNU alone (p < 0.01). A single i.p. injection of CCNU (50 mg/kg) was given 9 days after tumor inoculation. Similar results were obtained in 2 subsequent experiments with a total of 35 mice/group; values represent the combined data. Survival rate of mice treated by CCNU plus TP-1 was significantly higher than was the survival rate of mice treated by CCNU alone (p < 0.01).

Synergistic Effect of TP-1 and Chemotherapy in Treatment of 3LL. 3LL is known to be resistant to most of the available chemotherapeutic agents. It is, however, relatively sensitive to the nitrosoureas, especially trans-1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (23). In preliminary experiments, we were looking for a chemotherapy schedule that will be only partially effective, i.e., will improve survival rate of tumor-bearing mice but not completely cure them. Such a schedule may reflect optimally the possible benefit of the thymic extract under study. It was found that a single i.p. injection of CCNU (50 mg/kg) given 9 days after tumor inoculation, improved significantly the survival rate of mice treated by CCNU alone (p < 0.01), completely free of disease 6 months after treatment. However, when treatment consisted of both CCNU and TP-1, a substantial and significant (p < 0.01) increase in cure rate was obtained, with 56% of the tumor-bearing animals being cured. It should be mentioned that tumor appearance was earlier in these experiments (mean, 9 days) as compared to the experiment presented in Chart 1. TP-1 treatment was started the day after tumor inoculation.

Effect of TP-1 in Preventing Lung Metastasis and Increasing Survival Rate of Mice with Resected Primary Tumor. 3LL metastasizes to lung. Resection of primary tumor by amputation of involved leg does not necessarily avoid the development of lung metastasis and may actually enhance their appearance (11). In preliminary experiments, we found that, when primary tumor was resected, the incidence of fatal lung metastasis increased as a function of tumor size on resection day.

For the following experiments, mice bearing 3LL were divided into 3 groups according to the diameter of the primary tumor on the day of resection: small (<0.7 mm; mean, 0.53 ± 0.03); medium (0.7 to 1.7 mm; mean, 1.27 ± 0.07); and large (>1.7 mm; mean, 3.9 ± 0.73). TP-1 treatment was started 1 day after tumor resection. It was found that TP-1 increased significantly the cure rate of mice with small or medium-sized tumors. With small tumors, cure rate by the combination of resection and TP-1 was 97% as compared to 65% by resection alone (p < 0.05). With medium-sized tumors, cure was 42% for resection alone but 73% for resection accompanied by TP-1 treatment (p < 0.05). On the other hand, no effect of either resection or its combination with TP-1 was observed in the group of animals with large tumors.

Effective Combination of Resection of Primary Tumor, Chemotherapy, and TP-1 in Treatment of 3LL. The experiments with combination of TP-1 and either chemotherapy or resection of primary tumor indicated a significant increase in cure rate of animals as compared to each modality alone. The obvious next step was to evaluate the efficacy of combination of the 3 modalities of treatment. To answer this question, we selected mice with tumor diameters of 0.7 to 1.7 mm. Resection of primary tumor and CCNU treatment were performed on the same day (17th day after inoculation), and TP-1 (4 mg/kg) was given twice weekly thereafter. In these experiments (Chart 5), the effects of amputation alone or amputation plus TP-1 were comparable to those in previous experiments (Chart 4). Interestingly, the combination of amputation and CCNU was significantly less effective than was amputation plus TP-1 and only
complete tumor cell eradication or only cell stasis is involved and by what mechanisms it is achieved. However, the data suggest that TP-1 might be more effective against the metastasis than against the primary tumor. Indeed, differences in the immunobiology of the primary tumor and its metastasis were described (5, 8, 10). Moreover, resection of primary 3LL was shown to cause immune-mediated enhanced growth of its metastasis (11). The possible role of TP-1 in combatting such immune enhancement of metastatic growth should be further explored.

The combination of TP-1 and chemotherapy was effective both with or without resection of primary tumor. Such a combination was shown to be effective in another experimental tumor model (Moloney leukemia) and using thymosin Fraction V (6) and in clinical trials, that of small-cell carcinoma of lung with thymosin (18) and gastrointestinal cancer with TP-1 (33). TP-1 was shown to counteract chemotherapy-induced immunosuppression in humans (33), and this effect may be important in the ultimate effect on survival.

3LL, the tumor model used by us in this study, has several characteristics which make it similar to human solid tumors, including metastatic spread and relative resistance to most of the available cytotoxic drugs. On the other hand, the basic differences between such a transplanted tumor model and naturally occurring human tumors should not be ignored. The model tumor cells are multiply transferred, perhaps not truly syngeneic, with corresponding unknown adaptation and selection changes in antigenic structure, cell metabolism, and growth kinetics. They are transplanted in relatively large numbers in a nonnatural site to a normal immunological environment of a host and cause death within days or weeks, thus allowing a different immunological adaptation than do the natural, slow-growing human tumors. Nevertheless, such models may give us a general indication of the potential value of a certain approach to therapy and enable analysis of its underlying mechanism.

REFERENCES


Effect of the Thymic Factor, Thymostimulin (TP-1), on the Survival Rate of Tumor-bearing Mice

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