Inhibition of Growth of Ehrlich Tumors in Swiss Mice by Diphtheria Toxin

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SUMMARY

The effect of diphtheria toxin on tumor cells was studied in mice with transplanted Ehrlich ascites or solid tumor. For ascites tumor, the results were assessed on the bases of the trend in body weight, the incidence of ascites on the 13th day after transplantation, and the survival time. For solid tumor, the results were assessed on the basis of the weights of the dissected tumors. The results obtained demonstrate inhibition of both ascites and solid tumors. Inhibition of ascites tumor was dose dependent but the higher doses were toxic. The treatment was most effective after i.p. administration and weakest after s.c. administration. The i.p. route permitted a highly significant increase in survival time. The effectiveness of s.c. administration was improved when a given amount of diphtheria toxin was divided over several administrations, one per day, rather than administered in a single dose. Inhibition of solid tumor by i.p., i.m., and s.c. treatment was approximately 70%. Some possible explanations for the difference in sensitivity between normal and cancer cells are discussed.

INTRODUCTION

DT[1] is a protein of about 62,000 daltons (4, 5, 9, 10) secreted by lysogenic strains of *Corynebacterium diphtheriae*. The active toxin catalyzes a reaction between NAD and Elongation Factor II to form nicotinamide and an enzymically inactive adenosine diphosphoribosyl derivative of Elongation Factor II (11, 13). This reaction inhibits cellular protein synthesis in eukaryotic organisms. Labeled DT injected into guinea pigs and rabbits has a half-life of several hr and reaches all the organs (2). DT powerfully inhibits protein synthesis which leads to cellular damage or death. It also affects the viral replication cycle in intoxicated cells (6).

The sensitivity of mammalian cells to DT varies over a wide range. Among rodentia the guinea pig is most sensitive and the mouse is least sensitive. Cell resistance to DT is conceivably linked to the degree of macromolecular uptake and to the cellular factors of self-defense against the action of microbial toxins. Presumably, among the most important of these factors may be the ability of cell membrane to block the entry of the toxin (16, 17), the activity of pinocytosis (14), and the degree of activation of DT within the cell.

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[1] The abbreviations used are: DT, diphtheria toxin; MLD, minimal lethal dose.

We postulated that the toxin-resistant mouse cell becomes much less resistant when modified by neoplastic features. The current study was undertaken to determine whether DT exerts any detectable antitumor activity on Ehrlich carcinomas in mice.

MATERIALS AND METHODS

Mice. Experiments were carried out on male Swiss albino (Charles River CD-1 COBS) mice weighing 20 to 22g. The animals were maintained in a thermostatically controlled room at $23 \pm 1°$ with a 12-hr light cycle. They were fed an ordinary pellet diet (Charles River 4 RF) and tap water *ad libitum*.

Determination of Antitumor Activity against Ehrlich Ascites Tumor. Ehrlich ascites tumor cells were maintained by serial transplantation every 8 days. The ascites tumor cells (1×10^6 cells/mouse) were implanted i.p. into the mice. Unless otherwise stated, treatment was initiated 24 hr after tumor implantation, the DT being given once daily for 2 consecutive days. The toxin was dissolved in 0.9% NaCl solution; all volumes injected were 0.2 ml or less. Unless otherwise stated, the animals were divided into groups of 20. In order to make it possible to evaluate different parameters, each group of 20 animals was divided into 2 subgroups of 10 animals each. In the 1st subgroup the effect of DT was evaluated on the basis of the incidence of ascites tumor at autopsy performed 13 days after implantation. In the 2nd subgroup the effect of DT was evaluated by taking into consideration the changes in body weight and the survival times of the animals observed up to 50 days after implantation. On the morning of 51st day the animals were sacrificed and examined for the presence of tumors.

The Tukey wholly-significant-difference method (18) was used for the multiple comparison among various experimental groups. The other statistical evaluations were performed using the Dunnett test (8). The survival rate was expressed as (mean survival time of treated group/mean survival time of untreated control group) $\times$ 100.

Determination of Antitumor Activity against Ehrlich Solid Tumor. Ascites tumor cells (6×10^6 cells/mouse) were implanted s.c. on the back of the neck region. The treatment dose schedule was the same as that of the ascites tumor. All mice were sacrificed 10 days after implantation of cells. The solid tumor was carefully dissected out and weighed. The wet weights of the treated groups were compared with that of untreated control group; the statistical analysis was performed using the Dunnett test (8). The

tumor inhibition rate was expressed as follows: (1 − mean tumor weight of treated group/mean tumor weight of untreated control group) × 100.

DT. A crude toxin, Lot 47-C (Sclavo Laboratories, Sienna 53100, Italy) containing 120 limes of flocculation/ml (1000 MLD guinea pig per ml), was used in these experiments.

Diphtheria Antitoxin. A horse purified antitoxin (Sclavo Laboratories) containing 1000 i.u./ml was used in 1 experiment.

RESULTS

The antitumor activity of DT was investigated in 5 different experiments. In Experiments 1 to 4, animals with ascites tumor were used, while in Experiment 5 the animals were carriers of solid tumors.

In Experiment 1, the antitumor effect of the administration of various doses of DT was investigated. One hundred twenty male mice were subdivided at random into 6 groups of 20 animals each; 5 groups were given Ehrlich ascites tumor cells i.p., while the 6th group consisted of normal animals. Of the 5 groups with tumor transplants, 4 were treated with DT and 1 with 0.9% NaCl solution (untreated control group). The normal group of animals not receiving a tumor transplant was also treated with 0.9% NaCl solution. The DT was administered i.p. at doses of 1, 3, 5, and 7 MLD guinea pig/mouse/day, once per day, for 2 consecutive days, beginning the treatment 24 hr after the transplantation of the tumor.

The changes in body weight of the animals are shown in Chart 1*A*. The animals were weighed on alternate days. In order to maintain rigorous statistical comparison, weight determinations were terminated in groups in which mortality reduced the number of animals to less than 5. Table 1 shows the statistical evaluation of body weight at various intervals after the tumor implant. The data reported show that the body weight gain is affected by the administration of DT in a dose-dependent manner. In the group treated with 1 MLD/mouse/day of DT, the body weight at 7 days after the transplant was 25.6 ± 0.8 (mean ± S.E.). This value is significantly lower ($p < 0.01$) than the body weight of the untreated control group which had a mean weight of 29.8 ± 0.8 g but not significantly different from the body weight of the normal group of mice (25.3 ± 0.5 g). The difference found between the groups is temporary, however. On the 13th day after implantation, the group treated with toxin reached a weight of 32.7 ± 1.3 g which was not different, therefore, from the 32.7 ± 1.6 g achieved by the untreated control group. For all the other groups treated with higher doses of DT, the body weight was increasingly lower than the untreated control group. For all the groups, the difference was significant both at the 7th and 13th day. When the groups treated with toxin were compared with the group of normal mice, it was observed that at a dose of 3 MLD/mouse/day the body weight was slightly, but not significantly, decreased; but at higher doses of 5 and 7 MLD/mouse/day the inhibition in body growth became more intense and was constantly significantly different. Furthermore, at the highest dose, neurological disorders such
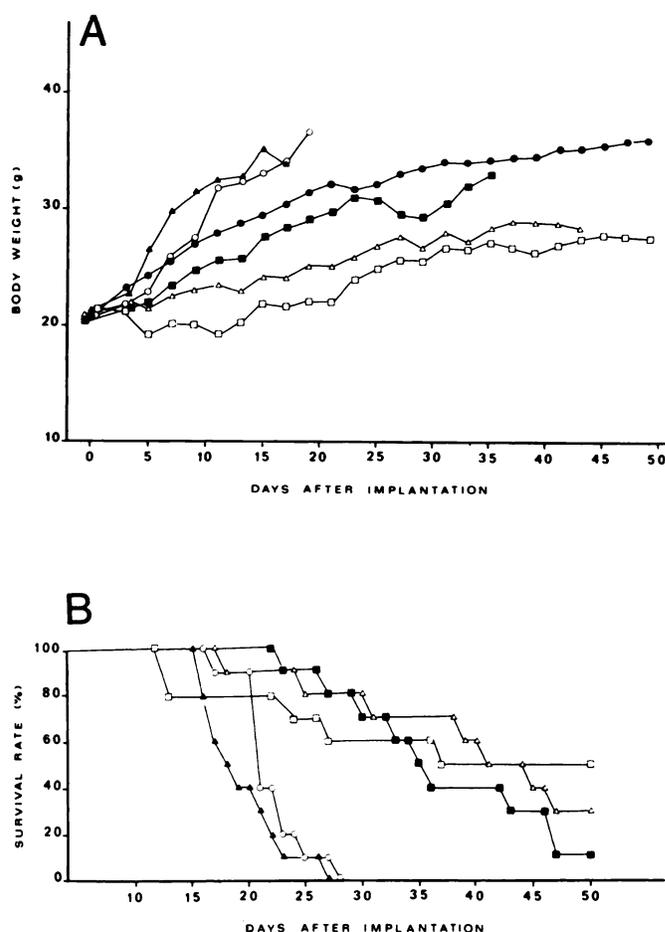

Chart 1. Antitumor activity of various doses of DT against Ehrlich ascites tumor. The DT was administered once daily for 2 consecutive days beginning 24 hr after the tumor transplant. Ten mice were used in each group. *A*, body weight; *B*, survival rate. ●, normal mice; ▲, untreated control; ○, DT, 1 MLD/mouse/day; ■, DT, 3 MLD/mouse/day; △, DT, 5 MLD/mouse/day; □, DT, 7 MLD/mouse/day.

as hind leg palsy and unidirectional circling became evident. Regarding the effect of DT on the incidence of ascites tumor revealed by autopsy on the 13th day, it was observed that, at the lowest dose (1 MLD/mouse/day), there was a 50% incidence of tumor; while for all the other groups treated with higher doses of toxin, no animal presented evidence of ascites tumor.

Chart 1*B* shows the survival curve and Table 1 shows the mean survival time. From the data obtained, it appears that the effect of DT on the survival of the ascites tumor-bearing animals is dose dependent. The most satisfactory results were obtained at the 2 intermediate doses, 3 and 5 MLD/mouse/day. In fact, whereas the animals of the untreated group had a mean survival time of 19.6 days, the animals treated with 3 and 5 MLD/mouse/day survived longer than 37 and 39 days, respectively. Also the animals treated with 7 MLD/mouse/day survived more than 36 days but showed signs of toxicity. Conversely, the dose of 1 MLD/mouse/day had only a weak influence on animal survival times. A total of 9 animals survived up to the 50th day in these experiments, all pertaining to the groups

Table 1
Antitumor activity of various doses of DT against Ehrlich ascites tumor

Body wt (g)	Normal mice	Tumor-bearing mice after following doses of diphtheria toxin[a]				
		Untreated control	1 MLD/ mouse/ day	3 MLD/ mouse/ day	5 MLD/ mouse/ day	7 MLD/ mouse/ day
Initial	20.8 ± 0.4[b] (10)	21.2 ± 0.3 (10)	20.9 ± 0.2 (10)	20.7 ± 0.3 (10)	20.9 ± 0.3 (10)	21.2 ± 0.5 (10)
Day 7[c]	25.3 ± 0.5[d] (10)	29.8 ± 0.8[e] (10)	25.6 ± 0.8[d] (10)	23.6 ± 0.4[d] (10)	22.4 ± 0.4[d, f] (10)	19.9 ± 0.8[d, e] (10)
Day 13	28.0 ± 0.5[g] (10)	32.7 ± 1.6[f] (10)	32.7 ± 1.3[f] (10)	25.8 ± 0.5[d] (10)	22.9 ± 0.6[d, f] (10)	20.2 ± 1.4[d, e] (8)
Day 21	32.2 ± 0.5 (10)			29.9 ± 1.1 (10)	25.1 ± 1.0[e] (9)	22.2 ± 2.1[e] (8)
Day 27	33.1 ± 0.7 (10)			29.7 ± 1.7 (8)	27.7 ± 0.9[f] (8)	25.7 ± 2.1[e] (6)
Day 35	34.5 ± 0.8 (10)			33.2 ± 2.0 (5)	28.6 ± 1.2[f] (7)	27.2 ± 1.9[e] (6)
Tumor incidence[h]		10/10	5/10	0/10	0/10	0/10
Mean survival time (days)		19.6	22.2	>37.1	>39.6	>36.3

[a] Treatment was initiated 24 hr after tumor implantation. The doses were administered i.p., once daily, for 2 consecutive days.
[b] Mean ± S.E. Numbers in parentheses represent the number of mice per group.
[c] Day 0 signifies the day of tumor implantation.
[d] $p < 0.01$, relative to untreated controls.
[e] $p < 0.01$, relative to normal mice.
[f] $p < 0.05$, relative to normal mice.
[g] $p < 0.05$, relative to untreated controls.
[h] Number of animals with evident ascites tumor at autopsy/total number of animals autopsied.

treated with DT. At autopsy, 1 animal showed no sign of tumor, while the other animals presented a solid s.c. abdominal tumor at the site in which the injections were made for the tumor transplant and the toxin treatment.

The 2nd experiment was designed to investigate the effect of DT on ascites tumor at various stages of development. One hundred male mice were given i.p. injections of Ehrlich ascites tumor cells and subdivided at random into 5 groups of 20 animals each. One of the 5 groups was treated with 0.9% NaCl solution and the other 4 groups were treated with DT. The DT was administered to all the groups at 3 MLD/mouse/day, once daily, for 2 consecutive days but the treatment was started at different days, *i.e.*, at the 1st, 2nd, 3rd, and 4th day after tumor implantation. One-half of the animals in each group were taken for autopsy on the 13th day. Chart 2 shows the survival curve. The results obtained demonstrate that the effectiveness of DT on survival of the animals with ascites tumor increased with earlier treatment. The longest survival time, greater than 39 days, was obtained in the group of animals in which treatment was started on the 1st day after transplantation of the tumor. The survival times for the groups treated 2, 3, and 4 days after transplant were greater than 30.7, greater than 30.7, and 18.9 days, respectively. Untreated controls survived 19.0 days. The incidence of ascites tumor in the animals autopsied on the 13th day also increased as the beginning of treatment was delayed. Tumor incidence was, respectively, 0, 30, 70, and 100% for the 1- to 4-day delay in treatment. Untreated controls showed 100% tumor incidence. At autopsy on the 51st day, 5 of the 7 surviving
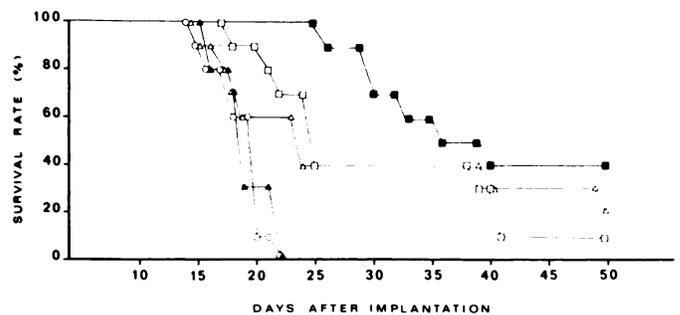

Chart 2. Antitumor activity of DT against Ehrlich ascites tumor in various degrees of development. The DT was administered i.p. at a dose of 3 MLD/mouse/day, once daily for 2 consecutive days. Ten mice were used in each group. ▲, untreated control; ■, treatment started 1 day after tumor implantation; □, treatment started 2 days after tumor implanation; △, treatment started 3 days after tumor implantation; ○, treatment started 4 days after tumor implantation.

animals presented a solid s.c. abdominal tumor at the injection sites. In 2 animals there was no evidence of solid or ascitic tumor. The 3rd experiment was designed to investigate the influence of the route of administration on the antitumor activity of DT. Eighty mice, subdivided into 4 groups of 20 animals each, were used. Three of the groups were treated with DT i.p., s.c., and i.m., respectively, at a dose of 3 MLD/mouse/day, once daily, for 2 consecutive days starting 24 hr after tumor transplant. From the results obtained (Chart 3) it appears evident that only the i.p. group demonstrated antitumor activity with a mean sur-

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vival time greater than 35.7 days. The mean survival times for the s.c.- and i.m.-treated groups were 22.1 and 21.6 days, respectively, not substantially different from the 19.2 days for the untreated group. The incidence of ascites tumor in the animals autopsied on Day 13 was 0% in the i.p.-treated group and 80 and 100% in the s.c.- and i.m.-treated groups, respectively. Untreated controls showed 100% tumor incidence. The 2 surviving mice in the group treated with DT i.p. showed no evidence of solid or ascitic tumor on final autopsy.

In Experiment 4, the effect of s.c. treatment was reconsidered, changing the dosage schedule of the DT. Forty mice were subdivided into 4 groups; 1 group acted as control and the other 3 groups were treated s.c. with DT, 6 MLD/mouse. Of these 3 groups, 1 received the DT in a single administration while the other 2 groups received the total dose of DT subdivided, respectively, into 3 (once daily for 3 consecutive days) and 4 (once daily for 4 consecutive days) administrations. For all groups the treatment was started 24 hr after transplantation of the tumor. Table 2 shows that the antitumor activity, although moderate, became evident only as the fractionation of the dose increased.

The 5th experiment was designed to determine whether the DT also possessed antitumor activity against Ehrlich solid tumor. The results obtained (Table 3) demonstrate that DT is capable of inhibiting solid tumor development by 79, 64, and 73% when administered i.p., s.c., and i.m., respectively. The differences in tumor weights in the groups treated with DT relative to the untreated control group were highly significant ($p < 0.01$).

A further experiment was performed to determine whether the antitumor activity of the crude toxin was specifically due to the DT. The results showed that the antitumor activity of crude DT was lost after incubation with horse antitoxin for 3 hr at 37° at a ratio of 1 MLD toxin/1 i.u. antitoxin.

DISCUSSION

Both Ehrlich ascites and solid tumor growths were inhibited by DT. Although a crude toxin was used in these experiments, the described effects were certainly toxin dependent since incubation with antitoxin rendered the crude DT completely ineffective. High doses of DT were required to produce both effective and toxic activity, since the mouse is the most toxin-resistant species among mammals. The inhibition of ascites tumor by DT was dose dependent, but increasing the dose produced signs of toxicity as evidenced by loss in body weight and appearance of neurological disorders. Conversely, the intermediate doses
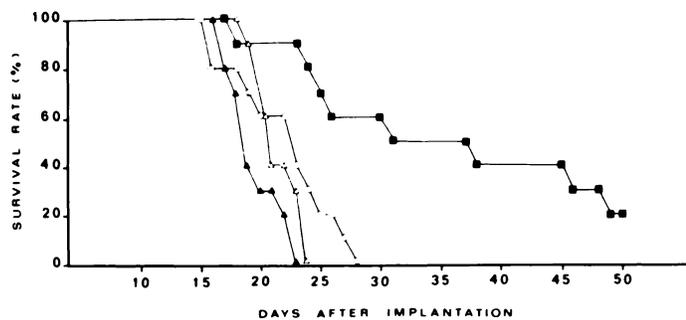

Chart 3. Antitumor activity of DT administered by different routes against Ehrlich ascites tumor. The DT was administered at a dose of 3 MLD/mouse/day once daily for 2 consecutive days. Treatment was begun 24 hr after the tumor transplant. Ten mice were used in each group. ▲, untreated control; ■, DT i.p.; O, DT s.c.; △, DT i.m.

Table 3
Antitumor activity of DT[a] against Ehrlich solid tumor

Route of administration	Tumor wt (mg)	Tumor inhibition rate (%)	p[b]
i.p.	409.5 ± 66[c]	79.1	<0.01
s.c.	701.5 ± 118	64.2	<0.01
i.m.	533.8 ± 100.5	72.8	<0.01
Untreated control	1962.6 ± 186	0	

[a] The DT was administered at a dose of 3 MLD/mouse/day, once daily, for 2 consecutive days. The treatment was initiated 24 hr after tumor implantation.
[b] Significance of the difference from untreated control values.
[c] Mean ± S.E.; 10 mice were used in each group.

Table 2
Effect of dose fractionation on the antitumor activity of DT

Body wt (g)	Untreated control	Diphtheria toxin[a]		
		1 dose fraction	3 dose fractions[b]	4 dose fractions[c]
Initial	22.2 ± 0.2[d]	22.4 ± 0.3	22.2 ± 0.2	22.4 ± 0.2
Day 9[e]	31.3 ± 0.9	32.5 ± 0.7	31.5 ± 0.9	25.8 ± 0.8[f]
Day 13	39.2 ± 0.9	38.0 ± 1.0	34.5 ± 0.9[f]	32.5 ± 1.2[f]
Day 17	38.6 ± 0.7	37.8 ± 0.8	36.4 ± 0.7	36.8 ± 1.4
Mean survival time (days)	19.9	20.0	21.1	26.3

[a] A total dose of 6 MLD/mouse was administered. The toxin was administered s.c. starting 24 hr after the tumor implantation. Ten mice were used in each group.
[b] DT was administered once daily for 3 consecutive days.
[c] DT was administered once daily for 4 consecutive days.
[d] Mean ± S.E.
[e] Day 0 signifies the day of tumor implantation.
[f] $p < 0.01$, relative to untreated controls.

showed evident antitumor activity without marked signs of toxicity. DT, in order to be effective in ascites tumor by the s.c. route, had to be administered over several fractional doses. It may be that fractional administration results in the maintenance of effective blood levels of DT for a longer period, thus affecting a greater number of cancer cells. Similar observations regarding toxicity were made by other workers in experiments with the guinea pig (15).

There are several possible mechanisms to explain the damage to cancer cells by suitable doses of DT. For example, firstly, both the cell membrane and the vessel endothelium may be more permeable in cancerous than in normal tissues, thereby causing an easier entry of DT. Secondly, the inhibition of protein synthesis mainly affects the cancer cells because of their high protein-metabolic activity. Thirdly, the initial binding of toxin to the cell may be electrostatic in nature. As both the toxin molecule and the cell carry a negative charge, the attachment of the toxin to the cell may be prevented. The binding of the toxin to the cell membrane is partially inhibited by polyanions and by high pH (7). Conversely, the binding is enhanced in the presence of salts (7). Thus cations may facilitate toxin binding to the cell by neutralization of some negative charges. Since the cancer cells have a relatively low pH, the hydrogen ions may facilitate the binding of the toxin to the cell membrane.

Finally, DT is a mixture of 2 similar proteins. One consists of intact polypeptide chains (intact toxin), while the other (nicked toxin) consists of 2 fragments of 24,000 and 38,000 daltons (A and B, respectively) linked to one another by a disulfide bridge (4, 5, 9, 10). Neither intact nor nicked DT has enzyme activity until after the disulfide bond holding A and B together is broken (4, 5, 10). Most of the activity of thiol-treated toxin was shown to be associated with a subunit of the toxin molecule (3) and precisely with Fragment A (4, 5, 10). Linkage to Fragment B is apparently required for toxicity, perhaps to facilitate entry of Fragment A into the cell (5). *In vivo*, the toxin may be reduced within cells through the action of —SH groups or other reducing agents, resulting in dissociation and hence release of active Fragment A (5). In the absence of thiol-reducing agents, trypsin also may hydrolyze the toxin molecule into the A and B fragments (9). We know that cancer cells are a good source of cathepsins (1, 12). Cathepsins are a mixture of proteases and among them cathepsin II is homospecific to trypsin (1). Thus, it may be that a greater content of cathepsins could activate DT to a greater extent in cancer than in normal cells.

ACKNOWLEDGMENTS

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