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Diphtheria Toxin Treatment of Human Advanced Cancer

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ABSTRACT

Purified diphtheria toxin was administered i.v. to 50 patients with advanced solid tumors. The doses of toxin varied according to the different immunological status of the patients against diphtheria. The prominent toxic effects of the treatment were transient peripheral neuropathy observed in two nonimmune patients and fever (37–41°) which occurred in all immune patients with cell-mediated hypersensitivity to toxin. A partial response lasting from 2 to 4 weeks was achieved in three of 13 nonimmune patients and in one of three nonimmune patients with cell-mediated hypersensitivity. Of 21 immune patients, nine had partial response lasting from 4 to 12 weeks. Of 13 immune patients with cell-mediated hypersensitivity, six had partial and five complete response lasting from 2 to 12 and from 1 to 25+ months, respectively. The overall response rate was 48%. The results suggest that diphtheria toxin may have a role in cancer immunotherapy.

INTRODUCTION

DT[1] is a M_r 62,000 protein released into the extracellular medium from virulent strains of *Corynebacterium diphtheriae*. The toxin consists of 2 unlike fragments, A and B, involved in different tasks. Fragment A is a potent inhibitor of protein synthesis in eukariotic cells; Fragment B mediates the attachment of the toxin to specific cell receptors found in several species including humans (10). Moreover, Fragment B is a strong immunogen and carries most of the antigenic determinants of the whole toxin molecule (12).

In recent years, the effect of DT in experimental tumors has been explored by several authors with conflicting results. Buzzi and Maistrello (5) showed that Ehrlich tumors regressed when mice were treated with DT. Iglewski and Rittenberg (8) confirmed this observation and reported that DT produced a greater inhibitory effect on the protein synthesis of mouse and human tumor cells than on their normal counterparts. Furthermore, Iglewski *et al.* (9) showed that Rous sarcoma virus-transformed cells and virus-induced tumors had increased susceptibility to DT. An immunological aspect of tumor treatment with DT was underscored by Buzzi and Buzzi (4) who reported that cancer immunity occurred in mice which were transplanted with DT-treated tumor cells. On the other hand, Pappenheimer and Randall (11) found that Ehrlich cells were not measurably more susceptible to DT than other mouse cells. Venter and Kaplan (15) reported that malignant human cells were not significantly more susceptible to DT than were normal cells, while Saelinger and Bonventre (13) showed that the malignant transformation of rodent cells did not alter their reactivity to the cytotoxic action of DT. Finally, Taylor and Iglewski (14), while confirming that virus-transformed cells were more susceptible to intoxication by DT than their normal counterparts, attributed the different responses to prior trypsinization of the cells.

In spite of the contradictory nature of the above reports, DT deserves careful consideration as a potential therapeutic agent in human cancer. In fact, beneficial effects against human tumors have been reported already by Buzzi (2, 3) after treatment of 11 patients with small doses of DT. That trial, however, had one major flaw. Only subjects with preexisting humoral immunity to DT were selected for treatment, while patients with other immunological status against diphtheria were excluded. By means of the Schick test, humans may be classified in the following immunological groups (7): (*a*) nonimmune nonhypersensitive subjects [individuals who lack humoral immunity and cell-mediated hypersensitivity to DT. They are highly susceptible to DT. It was calculated (1) that a nonimmune child could be killed by a dose of DT of only 1 MLD/kg body weight]; (*b*) nonimmune hypersensitive subjects (individuals who lack humoral immunity, while showing cell-mediated hypersensitivity); (*c*) immune nonhypersensitive subjects (individuals with only humoral immunity); and (*d*) immune hypersensitive subjects (individuals with humoral immunity and cell-mediated hypersensitivity from acquired infections or from immunization). The preexisting immunological status against diphtheria may influence the effect of the DT treatment in cancer patients. The cytotoxic activity of the protein should be more effective in nonimmune patients who cannot neutralize DT with specific antibodies. Conversely, the immunostimulating property should be more effective in immune patients who can react readily to DT.

This trial was undertaken to study the feasibility of DT treatment in human cancer and the effects of DT in cancer patients with different immunological status against diphtheria.

MATERIALS AND METHODS

Patients. Prerequisites for patient accession were: advanced histologically confirmed cancer; no other treatment in the 4 weeks before or during DT treatment; clearly measurable tumor for evaluation of the response (hepatomegaly was not acceptable); no severe malnutrition; estimated survival expectancy of at least 2 months; and informed consent.

Fifty patients were included in this study and their characteristics are shown in Table 1.

DT. A purified DT (Lot D 356–1; Connaught Laboratories, Toronto, Canada) containing 3000 Lf/ml, 35 MLD/Lf, and 0.018 μg nitrogen per MLD was used. After sterilization through a 0.2-μm membrane filter, DT was diluted with buffered NaCl solution [in g/liter of double-distilled water: NaCl, 8.5; KH_2PO_4, 0.27; $Na_2HPO_4 \cdot 2H_2O$, 0.83 (pH 7.3)] and then dispensed, at 2 different doses, in glass vials which were stored at −20° until used. After thawing and stirring, in 1 ml of the 2 different solutions were available 1 and 50 MLD of DT, respectively. These preparations maintained high stability for at least 6 months.

Clinical Studies and DT Administration. Baseline studies before

[1] The abbreviations used are: DT, diphtheria toxin; MLD, minimal lethal dose for guinea pig; Lf, limit of flocculation (quantity of toxin that flocculates most rapidly when mixed with 1 unit of antitoxin).

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the initiation of treatment included full blood counts, renal and liver functions, urinalysis, Mantoux skin test (0.1 ml of 1/1000 purified protein derivative of tuberculin solution; Sclavo Laboratories, Siena, Italy), immunoglobulin measurements (radial immunodiffusion plates; Behringwerke AG, Marburg/Lahn, Germany), and electrocardiographic and neurological examinations. In each patient, the immunological status against diphtheria was investigated by the Schick test and by the assessment of the titer of diphtheria antitoxin per ml of serum (Table 2). The pretreatment immunological assays were repeated 30 days after the treatment.

The Schick test was performed by i.d. injection of 0.1 ml of intact DT (0.02 MLD) in one forearm (the solution was obtained by diluting 1 ml of DT containing 1 MLD with 4 ml of buffered NaCl solution) and of 0.1 ml of heat-inactivated DT [boiling-water bath for 10 min (6)] in the other. Reaction to intact DT was read at 96 to 120 hr. A round deep-red inflammatory area slightly raised above the surrounding skin, sometimes tending to blister formation and persisting for many days (>5), indicated the lack of humoral immunity to DT (positive Schick test). The absence of such reaction indicated humoral immunity to DT (negative Schick test). Reaction to heat-inactivated DT was read at 24 to 48 hr. A short-lasting (<5 days), sometimes not sharply circumscribed, erythematous response of at least 1 cm in diameter to both inactivated and intact DT indicated cell-mediated hypersensitivity to DT (negative Schick test with pseudoreaction). A short-lasting response to inactivated DT and a long-lasting response to intact DT indicated a combined reaction (positive Schick test with pseudoreaction).

Table 1

DT treatment of cancer: patient characteristics

Total no. of patients	50
Age in years	
Median	66
Range	41–85
Sex (males/females)	28/22
Performance status	
Ambulatory	34
Partially bedridden	8
Bedridden	8
Primary tumor	
Lung	23
Breast	7
Stomach	6
Colon	5
Head and neck	5
Pancreas	1
Kidney	1
Bladder	1
Melanoma	1
Measurable tumor	
Palpable masses	26
Chest radiographs	33
Bone radiographs	2
Prior treatment	
Chemotherapy	10
Radiotherapy	2
Both	2
None	38

Table 2

Patient immunological status against diphtheria

The immunological reactivity of the patients to DT was studied by the titration of their circulating diphtheria antitoxin with an *in vivo* method and by the i.d. Schick test. The test detects the presence or the absence of humoral immunity and of cell-mediated hypersensitivity to DT

	No. of patients at each Schick test case			
Serum antitoxin titer (units/ml)	Nonimmune nonhypersensitive	Nonimmune hypersensitive	Immune nonhypersensitive	Immune hypersensitive
0	13	3		
0.005			6	1
0.01			6	2
0.02			5	3
0.04			4	5
0.08				2
Total	13 (26%)	3 (6%)	21 (42%)	13 (26%)

Table 3

Dosages of DT according to immunological status against diphtheria

As a rule, the daily dose of DT was administered i.v. for 3 consecutive days. Three nonimmune patients were treated with 0.02 MLD/kg/day every 5 days for 3 times. The ratio between the amount of DT administered to immune patients in a 3-day course and the amount of DT that their serum antitoxin could neutralize was greater than 1:2.

Patient serum antitoxin		DT dose (MLD/day)	
Titer (units/ml)	Total neutralizing capacity (MLD)	Nonimmune patients	Immune patients
0	0	0.02/kg (10)[a] 0.04/kg (6)	
0.005	≥350		50 (7)
0.01	≥700		100 (8)
0.02	≥1400		200 (8)
0.04	≥2800		400 (9)
0.08	≥5600		400 (2)

[a] Numbers in parentheses, number of patients at each dose.

The titer of serum antitoxin was studied by adding a constant amount of DT (0.05 ml of a solution obtained by diluting 1 ml of DT containing 1 MLD with 4.7 ml of buffered NaCl solution) to each of a row of tubes, the first of which contained 0.05 ml of undiluted serum of the patient and the others an equal volume of serum at doubling dilutions ranging from 1:2 to 1:64. After 30-min incubation at 37°, the mixtures were injected into the depilated skin of adult New Zealand rabbits. At 72 hr from the injections, the maximal dilution which prevented the inflammatory reaction of the skin was recorded. The antitoxin titer/ml, expressed in units relative to the international standard, was calculated by the formula

$$0.00025 \times 20 \times d$$

where 0.00025 is the constant amount of DT in Lf, 20 is the multiplicative factor to pass from 0.05 ml to 1 ml of serum, and d is the reciprocal of the maximal serum dilution which prevented inflammation.

Patients received different doses of DT according to their immunological status against diphtheria (Table 3).

Nonimmune patients (hypersensitive or not) were given i.v. either 0.02 or 0.04 MLD/kg/day for 3 consecutive days. Three cases were treated with 0.02 MLD/kg/day every 5 days for 3 times.

Immune patients (hypersensitive or not) were given i.v. 10 MLD/0.001 of antitoxin unit contained in 1 ml of their serum once a day for 3 consecutive days. Two patients were treated on alternate days. The daily dose was studied in order to give a total quantity of DT approaching but not exceeding the total amount that the serum antitoxin of the patient could neutralize. The ratio between the 2 values was greater than 1:2. The total amount of DT neutralizable by the patient was assessed approximately by the formula

$$a \times b \times 35$$

where a is the titer of serum antitoxin per ml, b is the total amount of serum per patient calculated as 4.5% of the body weight and expressed in ml, and 35 is the number of MLD which could be neutralized by 1 unit of antitoxin. The antitoxin contained in the intercellular space was not considered. In order to limit the quantity of foreign protein administered to patients, individuals with antitoxin titer greater than 0.04 unit/ml were treated with the same dose which was used at this level.

Response Criteria. The tumor size was measured by ruler or caliper either in 2 dimensions (metastatic pulmonary nodules, lymph nodes,

and s.c. masses) or in one dimension (mediastinal, hilar, and abdominal masses). A partial response was judged in the following cases: a 50% or greater decrease in the area of a bidimensional lesion (product of the 2 largest perpendicular diameters); a 50% or greater decrease in the sum of the areas of multiple bidimensional lesions; a 50% or greater decrease in the linear measurement of unidimensional lesions; and a decrease in size of lytic bone lesions. A complete response was given when all measurable lesions disappeared. Tumor sizes were measured before the treatment and weekly, for 8 weeks, after the first injection of DT. Thereafter, measurements were taken at 4-week intervals.

RESULTS

Toxic effects, tumor responses, and some clinical details of the responders in the different groups are summarized in Tables 4, 5, and 6, respectively.

Effects in Nonimmune Nonhypersensitive Patients. Thirteen of the patients (26%) pertained to this group. After treatment, a transient fall in systolic blood pressure (>25%) and asthenia were observed in 2 of the patients in this group. Two of the 3 subjects given injections of 0.02 MLD/kg/day every 5 days for 3 times developed peripheral neuropathy 40 days after the start of the treatment. These patients (a 74-year-old man with squamous carcinoma of the lip and a 71-year-old woman with abdominal metastasis of operated gastric cancer) complained of numbness and tingling of both hands and feet. Proprioceptive and sensory loss was noted in the fingers and toes. The neurological symptoms disappeared within 6 weeks.

Table 4

Toxic effects of DT treatment

Peripheral neuropathy involving hands and feet developed 40 days after treatment. Hypotension and asthenia lasted about 1 week. Fever, sometimes preceded by chill, lasted many hr and faded slowly with sweating.

	No. of patients affected			
	0.02–0.04 MLD/kg/day		10 MLD/0.001 antitoxin unit/1 ml serum/day	
Toxic effect	Nonimmune nonhypersensitive (13)[a]	Nonimmune hypersensitive (3)	Immune nonhypersensitive (21)	Immune hypersensitive (13)
Peripheral neuropathy	2	0	0	0
Hypotension, asthenia	2	1	6	9
Fever				
37–38°	0	1	0	5
38.1–39°	0	0	1	5
39.1–41°	0	0	0	3

[a] Numbers in parentheses, number of patients in each group.

Table 5

Response to DT treatment according to tumor type and immunological status against diphtheria

Measurements of tumor sizes were recorded before the treatment and weekly, for 8 weeks, after the first administration of DT. Thereafter, measurements were taken at 4-week intervals.

	No. of patients responding/total no. of patients			
Tumor type	Nonimmune nonhypersensitive	Nonimmune hypersensitive	Immune nonhypersensitive	Immune hypersensitive
Lung				
Squamous	0/2[a]		1/7	1/1
Adeno	0/1[a]		1/3	3/3
Small cell		1/1[b]	1/1	2/2[c]
Large cell				1/2[c]
Breast		0/1[b]	2/3	3/3[d]
Stomach	0/2[b, e]		3/4	
Colon	0/3[e]		1/1	1/1[c]
Head and neck	0/2[e]	0/1[b]	0/1	0/1
Pancreas			0/1	
Kidney	1/1[b]			
Bladder	1/1[b]			
Melanoma	1/1[e]			
Total	3/13 (23%)[f]	1/3 (33%)	9/21 (43%)	11/13 (85%)

[a] 0.02 MLD/kg/day for 3 consecutive days.
[b] 0.04 MLD/kg/day for 3 consecutive days.
[c] Complete response.
[d] Two partial and one complete response.
[e] 0.02 MLD/kg/day every 5 days for 3 times.
[f] The overall response rate was 48%.

Table 6

Clinical details of responders to DT treatment

						Response	
Patient	Group	Age	Sex	Primary tumor	Measurable tumor	Reduction (%)	Duration
1	NINH[a]	48	F	Kidney	Mediastinal and supraclavicular nodes	50–80	4 wk
2	NINH	74	M	Bladder	Lung	50	4 wk
3	NINH	54	F	Melanoma	Axillary nodes	60	2 wk
4	NIH	58	M	Lung (SC)	Lung	60	2 wk
5	INH	83	M	Lung (SQC)	Lung	60	12 wk
6	INH	65	M	Lung (AD)	Lung, cervical nodes	60	4 wk
7	INH	63	M	Lung (SC)	Lung, cervical nodes	60	4 wk
8	INH	83	F	Breast	Breast	50	8 wk
9	INH	62	F	Breast	Supraclavicular nodes	80	4 wk
10	INH	74	F	Stomach	Abdominal wall	50	4 wk
11	INH	47	M	Stomach	Abdominal wall	80	8 wk
12	INH	80	F	Stomach	Supraclavicular node	60	8 wk
13	INH	43	M	Colon	Lungs	50	4 wk
14	IH	49	M	Lung (SQC)	Lung	50	2 mos.
15	IH	64	M	Lung (AD)	Lung	60	4 mos.
16	IH	56	M	Lung (AD)	Lung	60	2 mos.
17	IH	50	M	Lung (AD)	Lung	60	3 mos.
18	IH	70	M	Lung (SC)	Lung	100	25 mos.+
19	IH	57	M	Lung (SC)	Lung	100	23 mos.+
20	IH	58	M	Lung (LC)	Lung	100	1 mo.
21	IH	82	F	Breast	Breast	70	6 mos.
22	IH	73	F	Breast	Femur, lung, pleura	50–100	12 mos.
23	IH	68	F	Breast	s.c. tissue, lung	100	4 mos.
24	IH	80	F	Colon	Abdominal wall	100	8 mos.

[a] NINH, nonimmune nonhypersensitive; NIH, nonimmune hypersensitive; INH, immune nonhypersensitive; IH, immune hypersensitive; SC, small cell; SQC, squamous cell; AD, adenocarcinoma; LC, large cell.

Three patients (23%) had a partial response of very short duration (2 to 4 weeks). Tumor regression in these patients became evident 5 to 7 days after the start of the treatment. When growth of the lesions recommenced, the responders were again treated with the same doses and the same schedule which was used in the first course, but no further response was achieved.

Effects in Nonimmune Hypersensitive Patients. Three of the patients (6%) pertained to this group. One of them had transient hypotension and fever (38°) arising 6 hr after the DT injections. Only this patient (33%) had a partial response of very short duration (2 weeks). When growth of the lesion started again, the treatment was repeated without benefit.

Effects in Immune Nonhypersensitive Patients. Twenty-one of the patients (42%) were included in this group. The most frequent toxic reactions induced by the treatment were moderate hypotension and asthenia which occurred in 6 patients for about 1 week. One patient (400 MLD/day) had a short-lasting fever (38.5°) arising 1 hr after the third injection of DT.

Nine of the patients (43%) had a partial response lasting from 4 to 12 weeks. The tumor regression was detectable from 7 to 30 days after the start of the treatment. Two patients of this series experienced a temporary enlargement of their lesions on chest radiographs before achieving a partial response 1 month after the treatment. The responders were again treated with DT when growth of the lesions restarted, but no further response was achieved. The doses of this second course were calculated on the basis of a new assessment of serum antitoxin of the patients.

Effects in Immune Hypersensitive Patients. Thirteen of the patients (26%) pertained to this group. All of them had from mild to severe reaction to the injected DT. After a lag of 6 to 12 hr from the injection, fever appeared ranging from 37° to 41°. In most cases, the high fever was preceded by chill, persisted for 6 to 12 hr, and faded slowly with profuse sweating. Two patients, who after the first injection of DT had fever lasting more than 24 hr, received the further doses on alternate days when their temperature had returned to normal values. One patient with squamous carcinoma of the tongue after the first dose had high fever which lasted 72 hr. DT treatment was discontinued and the fever was treated with aspirin. Altogether 9 patients were also affected by hypotension and asthenia lasting 1 week. During the week of the treatment, some tumor symptoms worsened greatly. For instance, cough became very distressing in 4 patients with lung cancer, while a woman with metastases of breast carcinoma to femur, lung, and pleura had intolerable bone pain and insistent dry cough.

There were 6 partial (46%) and 5 complete (38%) responses lasting from 2 to 12 and from 1 to 25+ months, respectively. Tumor regression was usually detectable 1 month after the start of the treatment. However, the last 2 cases of this series (Table 6) had a very slow regression of their tumor and achieved complete response only 3 months after the treatment. The degree of response was not related to the severity of the fever. Good regressions were obtained also with low (37–38°) or moderate (38.1–39°) fever. The responders were again treated with DT when growth of the lesions restarted, but no further benefit was obtained. The patients with long-lasting (>6 months) remission were given a new DT course every 6 months. The doses of each further course were calculated on the basis of a new assessment of serum antitoxin of the subjects.

Table 7
Immunological reactivity to DT of patients with different antitumor response

	Immune hypersensitive patients	
	Serum diphtheria antitoxin (units/ml)	Cell-mediated skin reaction to DT (mm)
Nonresponders (2)[a]		
BT[b]	0.03 ± 0.01[c]	12.5 ± 2.5
AT	0.06 ± 0.02	12.5 ± 2.5
Partial responders (6)		
BT	0.036 ± 0.01	15.0 ± 1.8
AT	0.06 ± 0.009[d]	30.0 ± 3.4[e]
Complete responders (5)		
BT	0.034 ± 0.01	16.0 ± 1.0
AT	0.06 ± 0.01	38.0 ± 3.4[f]

[a] Numbers in parentheses, number of patients in each group.
[b] BT, before treatment; AT, after treatment (30 days).
[c] Mean ± S.E.
[d] $p < 0.05$ as compared to pretreatment value (*t* test).
[e] $p < 0.01$ as compared to pretreatment value (*t* test).
[f] $p < 0.001$ as compared to pretreatment value (*t* test).

Changes in Immunological Determinants. The mean total circulating lymphocytes showed only minimal changes after DT treatment, while there was no change in cutaneous reactivity to purified protein derivative of tuberculin. IgG, IgA, and IgM levels, as well as the titer of serum diphtheria antitoxin, were raised in patients with immunocompetence against diphtheria. A correlation was found (Table 7) between a posttreatment increase of cutaneous cell-mediated reactivity to DT and the tumor response.

DISCUSSION

The feasibility of using DT to treat cancer in humans, the possible therapeutic activity of DT, and its effectiveness in diphtheria-nonimmune *versus* -immune subjects were the main issues of this work.

The most prominent toxic effects of the treatment were a transient peripheral neuropathy and a systemic delayed reaction to DT. The former occurred only in 2 nonimmune patients, while the latter was observed in all the immune hypersensitive patients. Both side effects, however, were generally well tolerated. Minor toxic effects of the treatment were hypotension and asthenia which were observed in all groups. In conclusion, DT treatment appeared to be feasible with tolerable clinical toxicity.

DT exerted antitumor activity in several patients. However, the immunological status of the subjects against diphtheria had a crucial influence in preventing or allowing the antitumor effect. While this effect was negligible in nonimmune patients, almost all of the responses were observed in immune and in immune hypersensitive groups. In particular, the immune hypersensitive group had the maximal rate of responses and included all the complete regressions which were obtained in this study. Furthermore, the patients of the same group who had antitumor response to the treatment also had a significant increase in cutaneous cell-mediated reactivity to DT. For the above reasons, it is conceivable that the antitumor effect of DT was dependent on the immunostimulating property of the toxin.

In this connection, it is of interest that 2 immune patients achieved a partial response after experiencing a temporary enlargement of their lesions on chest radiographs and that 5 immune hypersensitive patients had, after treatment, a transient worsening of tumor symptoms such as cough and bone pain. Both of these phenomena could be interpreted as being dependent on edema and inflammation of the neoplastic masses.

The mechanism which underlies the antitumor effect of DT is unknown. It may be that DT exerts a nonspecific stimulation of the host immune response as do other products of various corynebacterial species. Moreover, DT may induce the formation of circulating toxin-antitoxin complexes which in part may become deposited in the wall of the tumor vessels leading to alteration in permeability and to inflammation which damages the malignant cells. Alternatively, it is possible that a part of the injected DT escapes neutralization by antitoxin in the bloodstream and reacts tightly with specific membrane receptors of the tumor cells. After its binding to the cell membrane, the B fragment of DT could remain accessible to circulating antitoxin and to sensitized lymphocytes. Both of these immunological agents may elicit reactive phenomena involving the tumor.

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