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Targeting of GM2-bearing tumor cells with the cytolytic *Clostridium perfringens* delta toxin

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The cytolytic *Clostridium perfringens* delta toxin lyses selectively cells which express ganglioside GM2. In this study, we investigated whether delta toxin can be used to characterize GM2 on tumor cell membranes and as an antitumor agent. The sensitivity to lysis by delta toxin of various murine and human malignant cell lines and also normal tissues was quantified using a ^{51}Cr-release assay. The cytotoxicity titers were correlated with the ^{125}I-labeled toxin binding capacity of sensitive and insensitive cells. Seven of eight human melanomas tested were lysed by the toxin and, of these, four were very sensitive (cytotoxicity titers below 12 ng of toxin). All neuroblastomas, gliomas and the retinoblastoma tested were lysed with 3–18 ng of toxin. Three of six carcinomas and one of two sarcomas were also very sensitive (cytotoxicity titers 0.6–15 ng) whereas leukemias and lymphoma cells were insensitive. Normal human tissues were insensitive (erythrocytes, skin fibroblasts) or poorly sensitive (brain, lung, spleen). The *in vivo* antitumor activity of delta toxin was tested in tumor-bearing mice. Daily intra-tumor injections of 0.5–1 mg of toxin for 4–5 days in carcinoma Me180- and melanoma A375-bearing nude mice, and neuroblastoma C1300-bearing A/J mice significantly inhibited tumor growth for 12–36 days. Intravenous administration of 100 ng of toxin per day for 5 days in Me180-bearing nude mice and C1300-bearing A/J mice gave significant inhibition of tumor growth only during the treatment period, and 10 injections of the same dose of toxin had no significant effect on SK-MEL28, a tumor lacking GM2.

Key words: Clostridium perfringens, delta toxin, GM2.

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Introduction

Ganglioside GM2 is a sialic acid glycosphingolipid found at trace levels in normal tissues. (Gangliosides are termed according to the nomenclature of Svennerholm.[1]) It has been estimated to comprise only 3.6% of the total gangliosides of adult brain.[2,3] Monoclonal antibodies directed to its carbohydrate moiety have also detected the expression of this ganglioside in malignant tumors of neuroendocrine or neuroectodermal origin[4,5] and in squamous cell carcinoma and adenocarcinoma of the lung.[6] GM2 expression is also associated with viral, X-irradiation or chemical transformation of cells.[7] Furthermore, vaccines containing purified GM2 have been shown to elicit GM2 antibodies in melanoma patients with control of melanoma growth.[8] Thus, it appears that ganglioside GM2 may be a marker of cell differentiation and a target for cancer diagnosis and therapy.

In previous studies the specific interaction of the cytolytic *Clostridium perfringens* delta toxin with membrane ganglioside GM2 on target cells was evidenced by (i) the inhibition of cytolysis after incubation of delta toxin with GM2,[9,10] (ii) the characterization of GM2 on the membrane of cells sensitive to the toxin and its absence in resistant cells,[11] and (iii) ^{125}I-labeled delta toxin binding to thin layer chromatograms of neuroblastoma gangliosides containing GM2.[11] Delta toxin, a 40 kDa protein excreted by type C and some type B *C. perfringens* strains, was known as a hemolysin only active on erythrocytes of even-toed ungulates (sheep, cattle, goat)[9] before we demonstrated its lytic activity on other eukaryotic cells including

human monocytes[12] and platelets,[13] and rabbit macrophages.[14] The lytic mechanism of the toxin is a multi-step process initiated by a rapid and irreversible toxin binding at the cell surface leading to cell swelling and membrane disruption.[10,15]

In order to determine the ability of delta toxin to be used as a tool for the characterization of GM2 on the membrane of tumor cells and as an antitumor agent, the present study reports the *in vitro* and *in vivo* cytolytic effect of delta toxin on malignant cell lines from various origins.

Materials and methods

Delta toxin

The toxin was purified as reported previously.[9] Its specific cytolytic activity was 325 000 hemolytic units (HU)/mg of protein. One HU is the amount of toxin needed to release the hemoglobin from 50% of a standardized sheep red blood cell suspension (6×10^8 cells/ml), at 37°C within 45 min. One HU is equivalent to 3 ng of protein.

[^{125}I]Delta toxin

Delta toxin was labeled by the chloramine T method as described previously.[10] The radioactive specific activity of the labeled toxin ranged from 2 to 3 μg of protein. As reported previously,[10–14] no significant loss of hemolytic activity was detected after labeling of the toxin.

Cell lines

The different cell lines used in this study were obtained from the American Type Culture Collection (ATCC) with the exception of melanoma HxG2 and carcinoma HxG26, which were provided by Dr H Azar (Veterans Hospital, Tampa, FL), and human neuroblastoma cell lines SB, NA and SA which were established from patients seen in our institution. Cell lines derived from normal human tissues were also obtained from ATCC as NBL lines (NBL for Naval Biosciences Laboratory, Oakland CA) without any other characterization. The cells were subcultured in media (Gibco, Grand Island, NY) containing inactivated fetal calf serum (Gibco) according to the instructions given in the ATCC catalog and incubated at 37°C under 5% CO_2 atmosphere.

^{51}Cr-release assay

Cells (about 5×10^6) were detached from tissue culture flasks with trypsin 0.25%–EDTA 0.05%, washed once with fresh medium and incubated with 200 μCi $Na_2{}^{51}CrO_4$ (Amersham) in 1 ml of medium for 2 h at 37°C in 5% CO_2 atmosphere. The cells were then washed three times with phosphate buffered saline (PBS) and resuspended in medium at a final concentration of 2.5×10^5 cells/ml. Cells (5×10^4 cells in 200 μl) were added in individual wells of 96-well microtiter plates and incubated with various concentrations of delta toxin for 18 h at 37°C. Afterwards, the microtiter plates were centrifuged at 400 *g* for 5 min and the radioactivity in the supernatants was measured in a gamma counter. Maximal ^{51}Cr release was determined by lysing cells with 20 μl of 10% Triton X-100. The percentage of cell lysis was calculated as follows:

$$\% \text{ of lysis} = \frac{\text{test } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}}{\text{maximal } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}} \times 100$$

The sensitivity of different cell lines to delta toxin was assessed by the cytotoxicity titer expressed as the amount of toxin (nanogram of protein) needed to obtain 50% cytolysis in the assay. Cell lines which released less than 10% of ^{51}Cr in the presence of delta toxin concentration equal or superior to 600 ng/well were considered insensitive to the toxin.

Toxin-binding assay

Toxin-binding assays were performed in 1.5 ml Eppendorf tubes with 10^6 cells in suspension in 0.5 ml of medium. Cells were then incubated for 30 min at 37°C with different concentration of [^{125}I]delta toxin (5.77×10^6 c.p.m./μg of protein) or [^{125}I]delta toxin plus an excess of unlabeled toxin (15 μg) added 10 min before the labeled toxin as described previously.[10] Cells were centrifuged at 20 000 *g* for 10 min and the supernatants were removed. The sedimented membranes and cells were washed once with 1 ml of cold PBS, and counted in a gamma counter. All assays were performed in triplicate. The residual binding measured in the presence of an excess of unlabeled toxin was taken to represent non-specific binding. Specific binding of delta toxin was defined as the difference between total and non-specific binding.

Animals

Male BALB/c athymic *nu/nu* mice (5 week old) were obtained from Life Sciences (St Petersburg, FL). They were specific pathogen-free and maintained on sterilized water and animal food *ad libitum*. Male, 7 week old A/J mice were obtained from NCI (National Cancer Institute, Bethesda, MD).

Effect of delta toxin on various mouse organs and blood components

Three A/J mice were each injected with 500 ng of delta toxin into the lateral tail vein. Two mice died within 5 min of the injection and the last one died 20 min later. Blood samples were obtained by heart puncture soon after death and necropsies were performed on all of the animals. Samples of lung, heart, liver, spleen, kidney and brain were obtained and placed in 10% buffered formalin. Determinations of total white and red cell counts, hemoglobin concentration and platelet counts in EDTA anticoagulated blood were performed using an automated cell counter Sysmex E5000 (Toa Medical Electrics Co., Kobe, Japan). Differential cell counts were performed manually from blood smears. Formalin-fixed organ samples were processed, paraffin-embedded and hematoxylin–eosin stained for observation under light microscopy. Three blood samples from untreated A/J mice were processed in the same manner as the treated animals.

Tumor inoculation

Human A-375 and SK-MEL28 melanoma and human Me180 carcinoma were inoculated into nude mice. Murine C1300 neuroblastoma cells were inoculated into syngeneic A/J mice. 10^7 viable cells/mouse in 0.2 ml RPMI 1640 medium (Gibco) were injected subcutaneously into one flank. The animals were used for *in vivo* experiments when the tumor size was approximately 5–7 mm in diameter.

Antitumor activity of delta toxin in tumor-bearing mice

Groups of five to eight animals were treated with one daily injection for 5–7 days of either 100 ng of delta toxin (in 100 μl of RPMI 1640 medium) intravenously or 0.5–1 μg of toxin (in 100 μl of RPMI 1640) intratumorally as specified in the text. Tumor size was estimated from vernier caliper measurements of width and length of the tumor every 2 days and the tumor volume was calculated using the following equation: width^2 × length/2. Data were expressed as relative tumor volume (tumor volume at given time/initial tumor volume).[16]

Biodistribution of [^{125}I]delta toxin in tumor-bearing mice

A/J mice bearing C1300 neuroblastoma and nude mice bearing SK-MEL28 melanoma were injected intravenously or intratumorally with 100 ng of [^{125}I]delta toxin (5×10^5 c.p.m.). Groups of three animals were sacrificed 30 min and 18 h later. Blood, liver, spleen, lung, kidney, hind leg muscle and tumor were collected and weighed. The biodistribution of labeled toxin was determined by counting the radioactivity of tissues in a gamma counter. Results were expressed as c.p.m./100 mg of tissue.

Statistics

Statistical significance of correlation between toxin binding to cell membrane and cell lysis was determined by the non-parametric test of correlation of Kendall.[17] Statistical significance of tumor growth inhibition was determined by Student's *t*-test. *P* values of less than 0.05 were considered significant. Experiments were conducted a minimum of three times.

Results

In vitro effect of delta toxin on cultured human cell lines

The cytolytic activity of delta toxin was tested *in vitro* on various cultured human cell lines. As shown in Table 1, seven of eight human melanomas tested were sensitive to the toxin: 50% cytolysis was obtained with amounts of toxin less than or equal to 12 ng for four melanomas; whereas three melanomas were poorly sensitive (50% cytolysis obtained in a range of 54–300 ng). SK-MEL28 melanoma was insensitive. All other cell lines of neuroectodermal origin tested (neuroblastomas, gliomas and retinoblastoma) were lysed by the toxin in a range of 3–18 ng with the exception of

Table 1. Delta toxin cytolytic effect on human cell lines

Cell lines	Cytotoxicity titer[a] (ng of delta toxin)
Melanomas	
SK-MEL31	3
A375	4
HxG2	6
SK-MEL24	12
HT144	54
Malm3M	150
RPM17951	300
SK-MEL28	—[b]
Neuroblastomas	
SB9	3
NA8	18
SA spheroid	18
IMR32	40
Gliomas	
U-373 MG	12
HS683	17
Retinoblastomas	
Y79	15
Carcinomas	
Me180	0.6
HeLa	1.8
HxG26	15
CAOV3	60
BT20	60
CAPAN-1	—
Sarcomas	
TE85	4
Ewing's sarcoma	—
Leukemias	
Molt4	240
K562	—
HL60	—
Lymphoma	
U937	—
Normal cells	
erythrocyte	—
skin fibroblasts CCD-57 Sk	—
brain HS129	45
lung HS412	60
spleen HS193	84
fetal spleen HS230 SP	12

[a] Mean of three separate experiments performed in triplicate. Standard errors were always less than 10%.
[b] The insensitivity of cell lines to concentrations of delta toxin superior to 600 ng/well is represented by dash lines.

neuroblastoma IMR32 (40 ng). This cell line, however, is a mixture of neuroblasts and fibroblast-like cells. Only the neuroblast cell population appeared to be sensitive to the toxin. Five of six carcinomas tested were lysed. The two carcinomas of the cervix (squamous cell Me180 and adenocarcinoma HeLa) were extremely sensitive (50% cytolysis with less than 2 ng), while the adenocarcinoma of the pancreas (CAPAN-1) was insensitive and the squamous cell carcinoma of the neck (HxG26) had intermediate sensitivity. Ovarian carcinoma CAOV3 and breast carcinoma BT20 were poorly sensitive. Osteosarcoma TE85 was very sensitive whereas the Ewing's sarcoma tested was insensitive.

The leukemias K562 and HL60, and the lymphoma U937 were insensitive while leukemia cell line Molt4 was poorly sensitive. Cultured cells of normal tissues used as controls were negative (erythrocytes and skin fibroblasts) or poorly sensitive (brain, lung and spleen). Fetal spleen, however, was seven times more sensitive than adult spleen.

In vitro effect of delta toxin on mouse and rat cultured cells

Mouse melanoma B16 was poorly sensitive to the toxin (Table 2), whereas neuroblastoma C1300 and fibrosarcoma WEHI164 were extremely sensitive (50% cytolysis obtained with less than 1 ng of toxin). Tumor necrosis factor (TNF) sensitive and TNF insensitive mouse fibroblast L929 cell lines were resistant. Rat breast cancer cells MTFLR1 and MTFLR4 were poorly sensitive and insensitive, respectively.

Binding of [^{125}I]delta toxin on cultured cell lines

The relative sensitivity of several cultured cell lines to delta toxin was correlated with their capacity to

Table 2. Cytolytic activity of delta toxin on mouse and rat cell lines

Cell lines	Cytotoxicity titer[a] (ng of delta toxin)
Mouse	
fibrosarcoma WEHI164	0.3
neuroblastoma C1300	0.6
melanoma B16	120
fibroblast L929 (TNF insensitive)	—[b]
fibroblast L929 (TNF sensitive)	—
erythrocyte	
Rat	
MTF7LRI breast	300
MTF7LR4 breast	—
erythrocyte	—

[a] Mean of three separate experiments performed in triplicate. Standard errors were always less than 10%.
[b] The insensitivity of cell lines to concentrations of delta toxin superior to 600 ng/well is represented by dash lines.

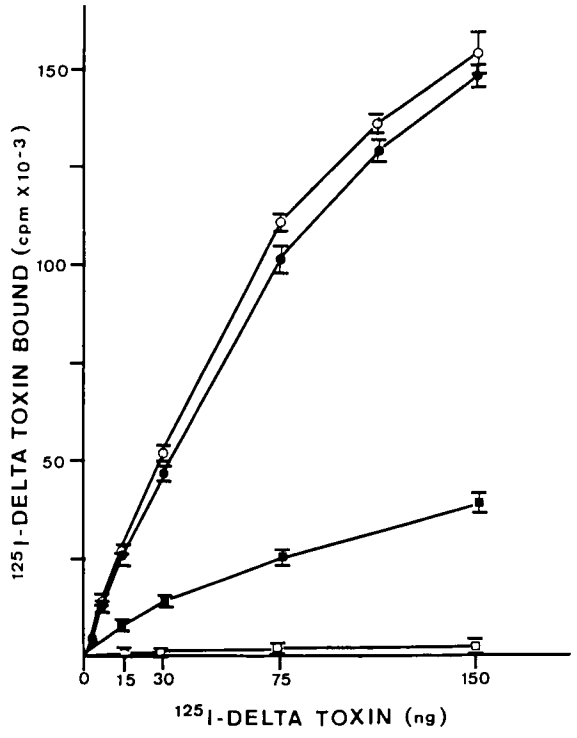

Figure 1. Binding of [^{125}I]delta toxin to different malignant cell lines. Between 3 and 150 ng of [^{125}I]delta was incubated with 10^6 cells of C1300 (○), Me180 (●) and SK-MEL28 (□) as described in Materials and methods. Bars represent standard deviations.

bind labeled toxin. Delta toxin bound significantly to C1300 and Me180 cells, and to a lesser extent to IMR32, whereas it did not bind to the resistant melanoma SK-MEL28 (Figure 1), as determined previously with erythrocytes and rabbit leukocytes.[10,13] This correlation was statistically significant ($p = 0.01$).

Toxicity of delta toxin in mice

The LD_{50} of the delta toxin was 185 ng when injected intravenously in A/J mice and nude mice. Death occurred within 30 min, probably from circulatory collapse and shock. Animals receiving sublethal doses were sick for 1 h and recovered. Daily intravenous injection of 100 ng of toxin for 5–7 days did not affect mice, which survived as long as control mice. Subcutaneous injections of toxin (2 μg) were not lethal. Blood and tissue samples obtained soon after death from A/J mice injected with a lethal dose of toxin (500 ng) showed no differences in white and red blood cell counts, hemoglobin concentration, and platelet numbers between control and delta toxin treated mice. In addition, no differences in the percentages of mononuclear and polymorphonuclear cells were seen in the blood smear differentials. Light microscopy observation of various organ samples from treated animals (lung, heart, liver, spleen, kidney and brain) showed no detectable morphological or inflammatory changes in any of the tissues examined.

In vivo antitumor effect of delta toxin

The ability of delta toxin to lyse tumor cells *in vivo* was studied by injecting non-lethal doses of delta toxin into tumor-bearing mice. Daily intratumor injections of 500 ng of toxin for 5 days significantly inhibited the growth of carcinoma Me180 (Figure 2) for up to 5 days after the end of treatment ($p = 0.0067$). After 36 days this effect was marginal

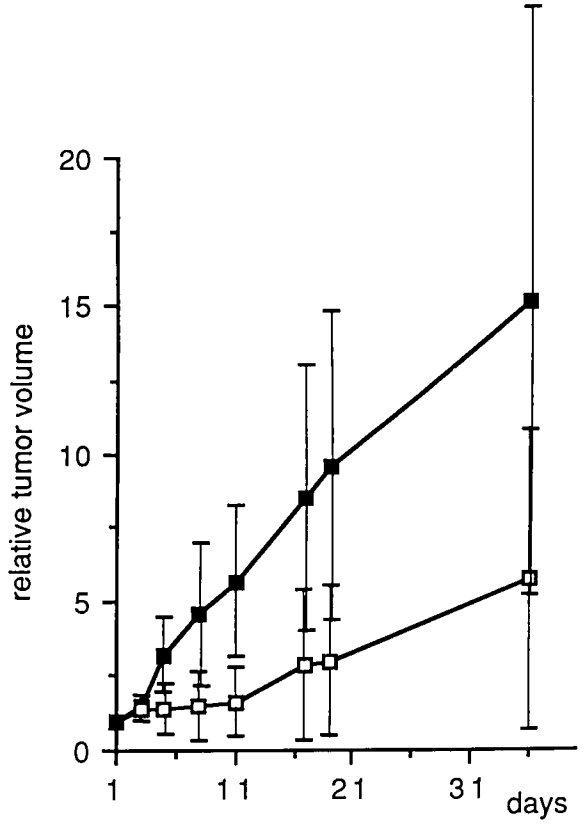

Figure 2. Inhibition of tumor growth after intratumor injections of delta toxin in carcinoma Me180-bearing nude mice. Groups of eight mice were injected for 5 days with either 100 μl of RPMI 1640 (■) or 500 ng of delta toxin diluted in 100 μl of RPMI 1640 (□). Squares represent the mean of the relative tumor volume and bars represent standard deviations.

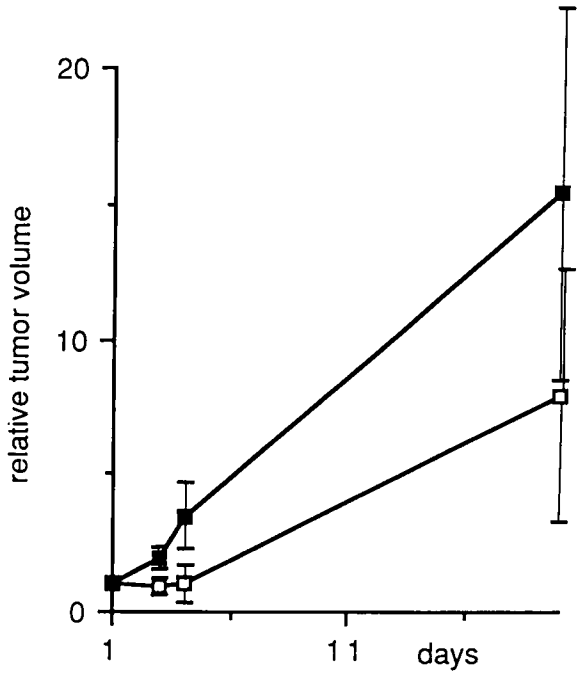

Figure 3. Inhibition of tumor growth after intratumor injections of delta toxin in melanoma A375-bearing nude mice. Groups of eight mice were injected for 4 days with either 100 μl of RPMI 1640 (■) or 1 μg of delta toxin diluted in 100 μl of RPMI 1640 (□). Squares represent the mean of relative tumor volume and bars represent standard deviations.

since the tumors of six out of eight treated animals continued to grow. However, the other two tumors disappeared with the treatment during this time. As shown in Figure 3, treatment of melanoma A375, another sensitive tumor, with 1 μg of toxin injected into the tumor daily for 4 days also caused initial inhibition of tumor growth. After 3 days of treatment, the difference of tumor growth between control and treated animals was significant ($p = 0.0002$) and six out of eight tumors decreased in size. A dose of 1 μg of toxin per day for 5 days also inhibited the growth of murine neuroblastoma C1300 (Figure 4). The inhibition of tumor growth was significant at 5 ($p = 0.0074$) and 7 ($p = 0.020$) days of growth. After 12 days, one out of five treated tumors totally disappeared and the difference in tumor growth between control and treated mice was marginal.

Intravenous delta toxin injection

Me180-bearing nude mice were injected intravenously daily for 5 days with 100 ng of delta toxin.

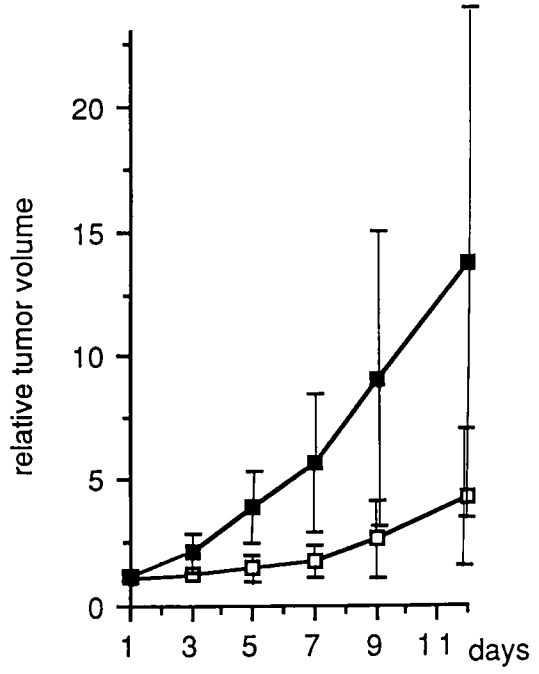

Figure 4. Inhibition of tumor growth after intratumor injections of delta toxin in neuroblastoma C1300-bearing A/J mice. Groups of five mice were injected for 7 days with either 100 μl of RPMI 1640 (■) or 1 μg of delta toxin diluted in 100 μl of RPMI 1640 (□). Squares represent the mean relative tumor volume and bars represent standard deviations.

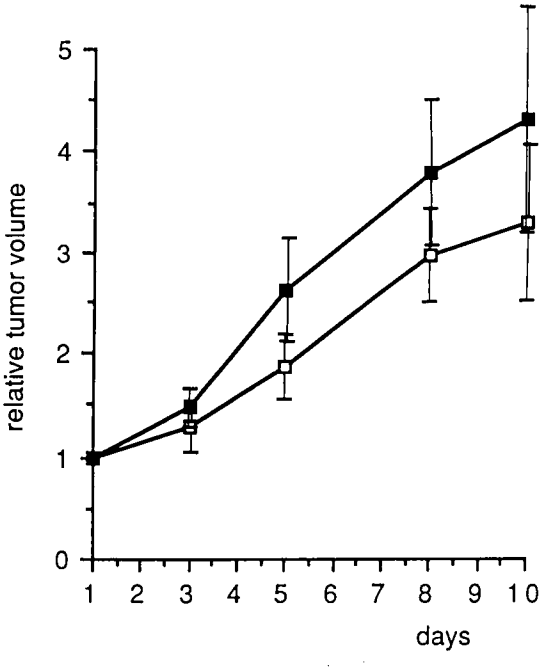

Figure 5. Inhibition of tumor growth after intravenous injections of delta toxin in carcinoma Me 180-bearing nude mice. Groups of five mice were injected for 5 days with either 100 μl of RPMI 1640 (■) or 100 ng of delta toxin diluted in 100 μl of RPMI 1640 (□). Squares represent the mean of relative tumor volume and bars represent standard deviations.

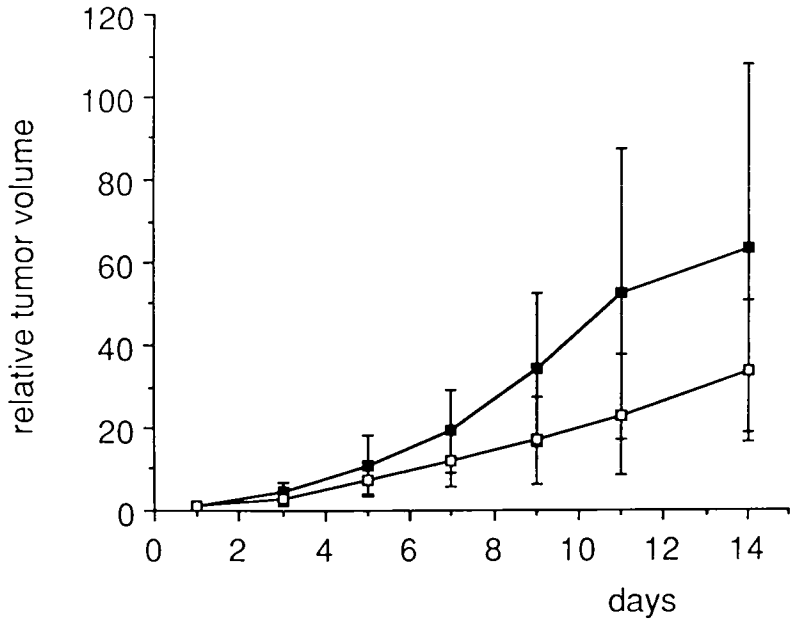

Figure 6. Inhibition of tumor growth after intravenous injection of delta toxin in neuroblastoma C1300-bearing A/J mice. Groups of seven mice were injected intravenously every other day with 5 doses of either 100 ng of delta toxin diluted in 100 μl of RPMI 1640 (□) or 100 μl of RMPI 1640 (■). Squares represent the mean of relative tumor volume and bars the standard deviations.

After 5 days, a significant inhibition of tumor growth could be observed in treated animals ($p = 0.035$) (Figure 5). However, the difference in tumor growth between treated and control animals became marginal thereafter. A/J mice bearing C1300 neuroblastoma tumors were injected intravenously every other day with 100 ng of delta toxin for 5 doses. In this experiment, inhibition of tumor growth was seen for up to 11 days from the beginning of treatment when compared with the control animals (Figure 6). As noted on mice bearing Me180 tumors, the difference in tumor growth decreased thereafter. As shown in Figure 7, no effect on tumor growth was seen in nude mice bearing the resistant SK-MEL28 melanoma after 10 doses of 100 ng delta toxin given every other day.

Biodistribution of [^{125}I]delta toxin in tumor-bearing mice

In order to assess the specificity of the antitumor effect of delta toxin in mice, [^{125}I]delta toxin was injected intratumorally in A/J mice bearing the sensitive C1300 tumor, and intravenously into mice bearing C1300 and SK-MEL28 tumors. As shown in Table 3, 31.7% of the injected dose of radioactivity remained in the C1300 tumor 18 h later, whereas other tissues were not significantly labeled. When injected intravenously in A/J mice bearing C1300 neuroblastoma and in nude mice bearing SK-MEL28 melanoma, [^{125}I]delta toxin was rapidly cleared from the blood circulation. After 30

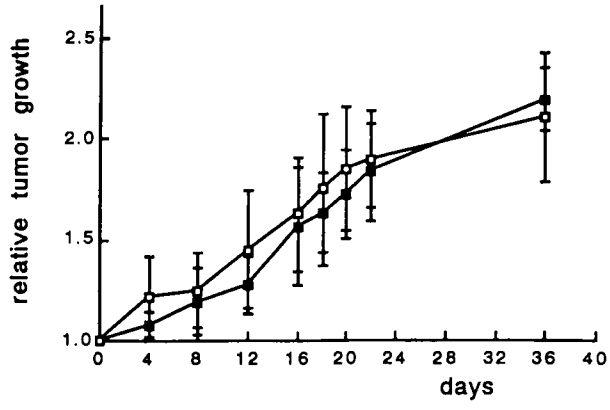

Figure 7. Inhibition of tumor growth after intravenous injections of delta toxin in SIC-MEL28-bearing nude mice. Groups of five mice were injected every other day with 10 doses of either 100 ng of delta toxin diluted in 100 μl of RPMI 1640 (□) or 100 μl of RPMI 1640 (■). Squares represent the mean of relative tumor volume and bars the standard deviations.

Table 3. Biodistribution of [^{125}I]delta toxin injected intratumorally into C1300-bearing A/J mice.

Tissues	Radioactivity in tissues[a,b]	Percent of total radioactivity[c]
Tumor	25158 ± 7365	31.7
Kidney	385 ± 204	0.19
Spleen	87 ± 8	0.02
Liver	93 ± 7	0.16
Lung	78 ± 22	0.02
Heart	28 ± 16	<0.01
Brain	14 ± 4	<0.01
Muscle	76 ± 15	ND[d]
Blood	21 ± 7	<0.01

[a] The c.p.m. of radioactivity/100 mg of tissue.
[b] Mean of results obtained with three mice ± standard error.
[c] Percent of total radioactivity remaining in the whole organs, blood and tumor after 18 h.
[d] ND, not determined.

min, only 16–20% of the injected dose of radioactivity remained in the blood, whereas high amounts of radioactivity were found in the lungs, kidneys, spleen and liver (Table 4). Radioactivity in these tissues decreased rapidly thereafter. In both systems, labeling of the tumor became maximal after 30 min and was stable for 6 h. At 18 h later, 42.5% of the maximal level of radioactivity was still found in the C1300 neuroblastoma while only 18.6% remained in the delta toxin resistant SK-MEL28 tumor. Lungs, spleen, kidneys and liver retained more radioactivity than the tumor, while blood, brain, heart and muscle contained lower amounts of radioactivity.

Discussion

By using the specific monoclonal antibody mAb 5-3 that detects ganglioside GM2, Natoli *et al.*[4] and Cheresh *et al.*[5] characterized the presence of this ganglioside in various cell lines. Our results show that delta toxin is as specific for GM2 as a monoclonal antibody. Cell lines which reacted positively with mAb 5-3 in immunoadherence and absorption assays (SK-MEL31, TE85, U373MG and B16) were sensitive to the lytic effect of delta toxin whereas non-reactive cell lines (SK-MEL28 and U937) were insensitive to the toxin. Furthermore, 50% cytotoxicity was obtained with the same amount of toxin (3 ng) for SK-MEL31 and TE85, which reacted to the same extent with mAb 5-3. The cytotoxicity titers of sensitive and insensitive cells significantly correlated with the respective amounts of labeled toxin that specifically bound to the same tumor cells. Thus, the expression of GM2 in the membrane of cells from various origins was quantified using these assays. GM2 was detected in seven of eight melanomas tested. As previously reported,[18,19] its expression is widely heterogeneous since there was a 100-fold difference in the sensitivities of melanomas SK-MEL31 and RPMI7951. No significant difference could be found between primary and metastatic melanomas. The other cell lines from neuroectodermal origin tested (neuroblastomas, gliomas and the retinoblastoma) were more homogeneous in their sensitivities to the toxin (ratio 3) with the exception of IMR32. This may be explained by the apparent mixed composition of this cell line (neuroblasts and

Table 4. Biodistribution of [^{125}I]delta toxin in C1300 neuroblastoma-bearing A/J mice and in SK-MEL28 melanoma-bearing nude mice after intravenous injection

Tissues	Radioactivity of tissues[a,b]					
	after 30 min		after 18 h		ratios[c]	
	C1300	MEL28	C1300	MEL28	C1300	MEL28
Blood	3964 ± 618	7561 ± 443	121 ± 32	596 ± 71	3.1	7.9
Liver	5026 ± 904	10942 ± 443	733 ± 92	1653 ± 64	14.6	15.1
Spleen	6398 ± 868	27481 ± 521	1984 ± 1734	7187 ± 477	31	26.2
Lung	6941 ± 2647	12038 ± 701	2108 ± 1507	2412 ± 186	30.4	20.0
Brain	117 ± 95	285 ± 27	116 ± 64	17 ± 5	100	6.1
Heart	4313 ± 13	5648 ± 794	118 ± 110	556 ± 44	2.7	9.8
Kidney	11033 ± 2045	12796 ± 473	1633 ± 321	1819 ± 174	14.8	14.2
Muscle	923 ± 107	901 ± 108	152 ± 78	231 ± 12	16.5	25.6
Tumor	1279 ± 154	1199 ± 87	557 ± 207	223 ± 51	42.5	18.6

[a] The c.p.m. of radioactivity/100 mg of tissue.
[b] Mean of results obtained with three mice ± standard deviation.
[c] Based on c.p.m. in tissues after 18 h c.p.m. in tissues after 30 min × 100.

fibroblast-like cells). Only the neuroblast cell population appeared to be sensitive to the toxin. The lytic activity of delta toxin on some carcinomas and sarcomas showed that GM2 expression is not restricted to tumors of neuroectodermal or neuroendocrine origin. Miyake *et al.*[6] generated two murine monoclonal antibodies that were able to discriminate *N*-glycolyl and *N*-acetyl neuraminic acid residues of GM2 and found that *N*-acetyl GM2 was present not only in small cell carcinoma tissues as one of the antigens related to tumors of neuroectodermal origin, but also in the squamous cell carcinoma of the lung with a comparable frequency. We have previously shown that delta toxin recognizes preferentially GM2 in its *N*-acetyl form.[11] The high sensitivity of cervic squamous cell carcinoma Me180, cervix adenocarcinoma HeLa and neck squamous cell carcinoma HxG26 to the toxin indicates that squamous cell carcinomas and adenocarcinomas of tissues other than lung can also express GM2.

Although GM2 has been reported to be a target structure on the K562 leukemia cell line for human natural killer cells,[20] this cell line as well as the U937 lymphoma (another target cell for natural killer activity) were not sensitive to delta toxin. Only GM2 extracts from K562 or melanoma MEL14 were able to inhibit the natural killer activity. These authors suggested possible structural differences in fatty acid lengths or in the composition of the ceramide portion of K562 GM2 to explain this observation. The insensitivity of K562, U937 and even Molt4, which was poorly sensitive to the toxin, supports this hypothesis.

It has been reported that the levels of gangliosides GM2 and GD2 increased in cultured melanoma cells as compared with the autologous tumor cells obtained directly from surgery or with cells grown in nude mice.[18,21] Although we did not perform comparable experiments with single cell suspensions, the cytolytic effect of delta toxin we observed *in vitro* on melanoma A375 was also evidenced *in vivo*. Intratumor injections of delta toxin in A375-bearing nude mice significantly inhibited the growth of the tumor during the period of treatment and for several days thereafter. This *in vivo* antitumor effect of delta toxin was also observed with the neuroblastoma C1300 and the carcinoma Me180, and correlated with the *in vitro* assay.

The injection of ^{125}I-labeled toxin in A/J mice evidenced uptake of toxin by lung, spleen, liver and kidneys. Although no detectable effects were observed on blood cells and tissues after injection of lethal doses of toxin in mice, the cytolytic activity of delta toxin on GM2-bearing cells of normal tissues may be the major cause of toxicity in mice. Daily intravenous injections of 100 ng of toxin for 5–7 days did not affect mice and also gave significant inhibition of tumor growth in Me180-bearing nude mice and in C1300-bearing A/J mice. This effect, however, was limited to the period of treatment. The low amounts of toxin injected intravenously and its wide tissue uptake may explain this limited effect. Previous studies on the antitumor effect of various immunotoxins in mice have also showed rapid clearance of these molecules from the blood, and uptake by liver, spleen and lungs.[22] Some authors have noted the relative inaccessibility of solid tumors to immunotoxins and suggested the use of vasoactive agents[23] or TNF[24] to alter the tumor/normal tissue perfusion ratio and thereby increase the effectiveness of tumor therapy.

The intratumor toxin treatment was more effective since the amount of toxin given to the mice could be increased to 1 μg per dose, and as evidenced with [^{125}I]labeled toxin, most of the toxin remained in the tumor. Higher doses (2 or 3 μg) were also injected but appeared to be lethal for some mice when their tumors were highly vascularized.

Many toxins have been conjugated to monoclonal antibodies directed to tumor-associated antigens in order to target their toxic effect to tumor cells. Such immunotoxins have been built with ricin, diphtheria toxin or pseudomonas exotoxin (see Pastan *et al.*[25] for a review). Their action, however, requires internalization of the antigen–antibody complex and all antigens do not mediate internalization. An immunotoxin has also been obtained by linking a surface-acting hemolytic toxin to an antigen expressed on immature T lymphocytes.[26] Work is in progress to build an immunotoxin that may enhance the delivery of delta toxin *in vivo* by using a monoclonal antibody directed against a specific determinant on GM2-bearing tumor cells. In theory, *in vivo* targeting of delta toxin may also be possible by using as carriers antigen-specific T lymphocytes or tumor-infiltrating lymphocytes transfected with delta toxin genes.[27] With its cytolytic activity targeted to tumor cells by its specific binding to membrane ganglioside GM2, and its lytic mechanism that occurs via the cell surface without internalization, delta toxin has a clear advantage over other natural toxins with antitumor activity as a potential tool for cancer therapy.

Prior to the clinical use of delta toxin, the precise distribution and density of GM2 in normal tissues needs to be determined in order to predict the

toxicity of the toxin in humans.[2,3,8] In this study, cell lines derived from normal human lung, brain and spleen tissues were poorly sensitive to delta toxin. In previous reports, however, we have shown a cytolytic effect of delta toxin on human platelets and monocytes.[12,13]

Based on the biodistribution data presented in this paper, it appears that the intravenous administration of delta toxin may be inefficient unless a carrier system is devised to deliver the toxin specifically to the tumor cells. Intratumor injections of delta toxin could, however, have some utility in the treatment of cutaneous melanoma. Regression of cutaneous metastatic melanoma has been observed in patients after intralesional injection with human monoclonal antibody to ganglioside GD2.[28] Since only 70% of all melanomas express GD2, and GD2-negative melanomas may express GM2, the authors considered that treatment with both monoclonal antibodies anti-GD2 and anti-GM2 could enhance the therapeutic effects on melanomas. A combination of intratumor injection of delta toxin with anti-GD2 antibody could have a cumulative antitumor effect and might offer some advantage over anti-GD2 treatment alone. Furthermore, the small size of the toxin (M_r 40 000) would reduce the access of antibodies to neoplastic cells.[29] Finally, only further investigation will determine the possibilities of delta toxin as an antitumor agent.

Conclusion

Delta toxin is a potentially useful tool for the diagnosis and treatment of cancer because of its specific affinity for ganglioside GM2, expressed in a variety of transformed cells. While delta toxin is extremely active *in vitro*, its antitumor effect *in vivo* is modest and occurs only during the treatment period because of toxin uptake by the reticuloendothelial system. However, delta toxin offers a clear advantage over other natural toxins for the construction of an immunotoxin since its antitumor effect does not require internalization. This delivery system may improve the targeting of delta toxin to GM-2 bearing tumor cells *in vivo*.

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