Selective Toxicity of Diphtheria Toxin for Malignant Cells
(cancer/protein synthesis)

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ABSTRACT Purified diphtheria toxin is shown to inhibit protein synthesis in Ehrlich–Lettre ascites carcinoma cells in vitro. Protein synthesis in Ehrlich–Lettre cells is at least 10,000 times more sensitive to toxin than protein synthesis in normal mouse spleen or thymus cells. This sensitivity correlates with the observation that Ehrlich–Lettre tumors regress in mice injected with diphtheria toxin but not diphtheria toxoid. Using the criterion of inhibition of protein synthesis in vitro, we show that other mouse malignancies (lymphoma and myeloma) are also more sensitive to diphtheria toxin than normal spleen or thymus. Metastatic human breast carcinoma cells from two individuals, cells from two melanoma nodules removed at different times from a third patient, and cells from melanoma nodules from three additional individuals are shown to be more sensitive to diphtheria toxin than some normal human cells. The toxin sensitivity of protein synthesis in some of the malignant cells tested was so much greater than that of normal cells, that we have suggested that diphtheria toxin should be studied further since it might prove a useful anti-cancer agent in patients whose tumors are first shown to be highly sensitive to toxin in vitro.

While studying specificity of mouse antibodies against highly purified diphtheria toxin or toxoid, we injected Ehrlich–Lettre ascites tumor cells into mice in order to induce ascitic fluid for harvesting large amounts of antibody. We observed regression of Ehrlich–Lettre ascites tumors (ELa) only in mice injected with toxin. Subsequently we found that this effect was caused by toxin and not by toxoid, even when all animals had been preimmunized with toxoid (Fig. 1). This observation suggested that while normal mouse tissues are relatively insensitive to diphtheria toxin (1, 2), ELa cells are sensitive to diphtheria toxin. Here we provide in vitro data confirming this hypothesis. We find that protein synthesis in ELa cells is at least 10,000 times more sensitive to diphtheria toxin than protein synthesis in normal mouse cells. Furthermore, we show by the criterion of inhibition of protein synthesis that other mouse tumor cells are sensitive to diphtheria toxin and that cells from different tumors show different degrees of sensitivity relative to normal cells.

We also present preliminary data indicating that protein synthesis in human breast carcinoma cells and human melanoma cells is more sensitive to diphtheria toxin than protein synthesis in cells from normal human breast or skin.

MATERIALS AND METHODS

Cells. Cell lines or tumors used in this study were generously provided by Dr. C. E. Wenner (ELa), Dr. D. Regan (6C3HED lymphoma), Mr. W. W. Muryaz, Cell Distribution Center, Salk Institute (myeloma C1), Dr. D. Tershak (HeLa cervical carcinoma), and Dr. D. McConnell (human melanoma, breast carcinoma, and normal skin). Normal human heart, pancreas, and breast tissue were obtained at autopsy. Mouse tumors were maintained by serial passage in female mice: ELa (Swiss–Webster), and lymphoma and myeloma (C3H). Spleen and thymus cells were obtained from normal Swiss–Webster, C3H, and Balb/c mice. HeLa cells were grown in Eagle’s minimal essential medium containing 10% (v/v) fetal calf serum and 1% glutamine (w/v).

Diphtheria Toxin was obtained from two sources: (1) purified and concentrated lot D279 [1600 L italiane units/mg of nitrogen], Connaught Medical Research Laboratories; (2) purified toxin (3000 Lf/mg of nitrogen), gift of Dr. R. J. Collier. A portion of the latter was treated with formalin to produce diphtheria toxoid (3).

Assay for Susceptibility to Diphtheria Toxin. Hanks’ minimal essential medium was modified to contain 1/100 the normal concentration of essential amino acids, 2% fetal calf serum, and 1% glutamine. Fetal calf serum was extensively dialyzed against Hanks’ balanced salt solution. Hanks’ modified minimal essential medium was used to wash and maintain

![Fig. 1. Effect of diphtheria toxin on growth of ELa cells. Ten mice in each of three groups were given subcutaneous injections of 3 μg of toxoid in complete Freund’s adjuvant at weekly intervals for 3 weeks. Mice were injected with 5 × 10⁵ ELa cells intraperitoneally 9 days after the final injection of toxoid. Five days later, mice received an intraperitoneal injection of (A) 3 μg of diphtheria toxoid, or (B) 3 μg of diphtheria toxin, or (C) diluent. Mice were observed and weighed daily. Representative individuals from each group were photographed 19 days after tumor injection.](image-url)
cells and as a diluent. All cells were washed twice immediately after harvest. Spleen cells were obtained as described (4). Thymocytes and myeloma cells were obtained by mincing the organ or tumor with scissors and gently pressing the tissue through a stainless steel tea strainer. Fragments were allowed to settle, and the supernatant cells were removed and centrifuged. Spleen, thymus, and myeloma cells were freed of erythrocytes by ammonium chloride (5). Ammonium chloride (NH₄Cl) at 3.0 μmol/ml inhibits the action of diphtheria toxin if incubated with the toxin and HeLa cells for 14 hr (6). In our experiments the cells were incubated with 0.07 μmol/ml of NH₄Cl for 5 min and then washed twice with Hanks' modified medium before addition of diphtheria toxin. Furthermore, a control experiment was done using toxin-sensitive HeLa cells to demonstrate that inhibitory amounts of ammonium ion were not present in the assay. HeLa cells treated with NH₄Cl as above showed the same sensitivity to diphtheria toxin as did HeLa cells treated with diluent. Normal human skin, human tumors, and mouse lymphoma were dispersed as described above for mouse thymus, but were not treated with ammonium chloride. Tumors grown as ascites were harvested as described (7). Cell monolayers were harvested with 0.125% (w/v) trypsin as described (8). Washed cells (4 X 10⁹) were seeded in screw-capped tubes with various concentrations of diphtheria toxin and incubated 3 hr at 37°.

Then 1 μCi of either ¹⁴C-labeled or ³H-labeled amino acid mixture (New England Nuclear Corp., NEC 445 or NET 250) was added to give a final volume of 1 ml. After 2 hr at 37°, cells were washed twice with phosphate-buffered saline (pH 7.2), frozen and thawed, and processed for counting of radioactivity in a Beckman LS 200 spectrometer (9). Percent inhibition of incorporation was determined from the mean of triplicate samples exposed to diphtheria toxin compared to triplicate samples from control cultures without the toxin. In our experiments, the average standard error of the mean is 5.8%, with a range of 0.4-17.8%.

RESULTS

As defined by inhibition of protein synthesis, ELa cells were at least 10,000 times more sensitive to diphtheria toxin than normal mouse spleen or thymus cells (Fig. 2). Protein synthesis in ELa was inhibited 60% by as little as 0.000 μg of toxin, while normal spleen or thymus cells were not inhibited by 30 μg of toxin. Similar results were obtained in six separate experiments. Myeloma C1 and lymphoma 6C3HED displayed intermediate sensitivity, more sensitive than normal tissue but less sensitive than ELa. A Balb/c myeloma tumor (MOPC 315) gave results identical to those for myeloma C1 (data not shown). The data shown are for the solid lymphomas, but the ascites form of the lymphoma responded similarly. Thus, growth in the peritoneal cavity in itself does not confer high sensitivity to toxin nor does sensitivity seem to correlate with growth of solid or dispersed tumor cells.

We next tested the sensitivity of protein synthesis in several human tumors to diphtheria toxin (Figs. 3 and 4). Cells from metastatic breast carcinomas of two separate patients were sensitive compared to normal breast cells or to skin cells from one of the two patients. Protein synthesis in cells from breast carcinoma no. 2 was inhibited 50% by 0.03 μg of diphtheria toxin, the same sensitivity displayed by HeLa cells at that dose. The cells from breast carcinoma no. 1 required 3.0 μg of diphtheria toxin for protein synthesis to be inhibited 50%. Nevertheless, this tumor also appears sensitive to diphtheria toxin when compared to cells from normal breast or skin. Microscopic examination of the malignant tissue samples indicated that they contained both malignant cells and some normal cells. In view of the contamination of malignant samples with normal cells, we tested the sensitivity of cells from adjacent tissue underlying carcinoma no. 1. Microscopic examination of this tissue showed it to contain many normal cells and fewer malignant cells. Cells from this sample showed intermediate sensitivity, suggesting that this method might be used to discriminate between normal and malignant cells. In these and in other tumor tissues examined it has been our experience that the steepness of the sensitivity curve is a reflection of the relative proportions of normal and malignant cells.

Fig. 4 shows that protein synthesis in cells from two melanoma nodules obtained on different days from the same individual is sensitive to diphtheria toxin. While the sensitivity curves are not identical, both show increased sensitivity to diphtheria toxin compared to normal skin cells from the same individual. Tumor cells and normal cells from three additional melanoma patients have shown similar relationships. Similar but less dramatic sensitivity is shown by cells from a small cell undifferentiated carcinoma of the neck from a different patient. This sample also contained a large proportion of normal cells. While we have not yet been able to complete an extensive survey comparing the diphtheria toxin sensitivity of protein synthesis in cells from other normal human organs, we
are also differential sensitivity to diphtheria toxin. The assay was performed as in legend of Fig. 2 except *H*-labeled amino-acid mixture (*aa*) was used (see Methods). (•—••) Cells from subcutaneous tissue adjacent to carcinoma (patient no. 1), (●—●) cells from a metastatic breast carcinoma (patient no. 1), (△—△) cells from a metastatic breast carcinoma (patient no. 2), (□—□) HeLa cells, (△—△) normal breast cells, (Δ—Δ) normal skin cells (patient no. 1). The results for normal skin cells were identical to normal breast cells at all concentrations of diphtheria toxin less than 60 μg.

have tested samples of normal human heart and pancreas, which are the most sensitive organs in the guinea pig (10). Human heart and pancreas cells behaved like skin cells in their diphtheria toxin sensitivity (data not shown).

DISCUSSION

Diphtheria toxin is a potent inhibitor of protein synthesis in mammalian cells. The toxin may be cleaved by some proteolytic enzymes into two polypeptide fragments, A and B (11, 12). The B portion of the molecule facilitates binding of diphtheria toxin to the cell membrane (13). Fragment A is an enzyme that inhibits protein synthesis by catalyzing the transfer of adenosine diphosphate ribose from NAD to elongation factor 2, thereby preventing continued growth of polypeptide chains (1, 14). Tissues or cultured cells of all animal species are not equally susceptible to diphtheria toxin (2). Since elongation factor 2 extracted from resistant cells is also sensitive to the enzyme activity of fragment A (1, 15), it is believed that resistance to diphtheria toxin is a property of the cell membrane. Resistance may reflect the absence or masking of cell receptors for the toxin or the failure of bound toxin to enter the cell (16). Although not yet proven, the differential sensitivity of protein synthesis in tumor cells may also be a property of the cell membrane.

Normal mice or cell lines derived from normal mouse tissues are highly resistant to diphtheria toxin (2, 17). Our results with mouse spleen and thymus cells (Fig. 2) confirm these earlier conclusions. The regression of ELa tumors in mice injected with diphtheria toxin (based on the weight of animals and visual observations) (Fig. 1), suggested that malignant transformation increases the sensitivity of mouse cells to diphtheria toxin. Protein synthesis in ELa tumor cells tested in vitro was at least 10,000 times more sensitive to diphtheria toxin than that of cells from normal mouse tissues (Fig. 2). During the preparation of this manuscript, Buzzi and Maistrello (18) reported that the development of Ehrlich tumors was indeed inhibited if mice were injected with crude diphtheria toxin at intervals after tumor inoculation. Although they carried out no experiments in vivo, they suggested that Ehrlich cells might be more sensitive to diphtheria toxin. Our observations confirm that diphtheria toxin will prevent in vivo development of ELa tumors, and our experiments in vitro show clearly that ELa cells and other mouse tumor cells are more sensitive to diphtheria toxin.

The in vitro experiments reported here leave no doubt that by the criterion of inhibition of protein synthesis some tumor cells are more sensitive to diphtheria toxin than some normal cells from the same species. Thus, in mice (Fig. 2), ELa (carcinoma) cells were the most sensitive to diphtheria toxin, lymphoma and myeloma cells displayed intermediate sensitivity, and normal spleen and thymus cells were relatively insensitive. Relative sensitivities among tumors, seen also in the human samples (Figs. 3 and 4), cannot at this time be ascribed to individual differences between the tumors themselves. As indicated above, our experience thus far suggests that the slopes of the sensitivity curves reflect the degree to which tumor cells are contaminated with normal cells, but this observation remains to be demonstrated quantitatively.
The lymphoma we used (6C3HED) has thymus-derived (T cell) characteristics (C. Bianco, personal communication) but is more sensitive to diphtheria toxin than normal thymus cells, suggesting that sensitivity to the toxin may not be explained on the basis of differences in the tissue of origin. A sufficient number of normal and neoplastic tissues have not been tested to permit the generalization that malignant transformation always confers increased sensitivity to diphtheria toxin. Nevertheless, this is a question worth pursuing. The simple protein synthesis assay used here might be used to detect the presence of malignant cells in biopsy specimens by comparing toxin sensitivity curves between suspect and normal tissues. Even more importantly, this in vitro assay might also be predictive of a tumor's responsiveness to toxin therapy in vivo. However, before these applications can be realized, a standard basis for making comparative judgments of sensitivity to diphtheria toxin must be devised. Such a standard will require correlation of percent inhibition of protein synthesis at a given concentration of diphtheria toxin to cell viability. Furthermore, an extensive survey of all normal human tissue types is required to enable comparison of the sensitivity to diphtheria toxin of a given tumor to the most sensitive normal human tissue.

The effect of diphtheria toxin on ELA cells compared to normal mouse cells vastly exceeds the therapeutic index of conventional cancer chemotherapeutic agents (19). We suggest that diphtheria toxin might be used for anticancer therapy in patients whose tumors are shown to be sensitive in vitro if our preliminary observations can be extended to other animal species and broadened to include additional human samples. While sublethal doses of toxin may themselves prove therapeutic, higher doses might be possible. In the latter case, individuals might be protected from generalized toxicity either by preimmunization or by concurrent administration of passive antitoxin antibody.

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