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Cell Biology

On the alleged high sensitivity of mouse Ehrlich-Lettre ascites tumor cells to diphtheria toxin

(protein synthesis)

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ABSTRACT **It was recently reported by Iglewski and Rittenberg in THESE PROCEEDINGS (71, 2707–2710, 1974) that low doses of purified diphtheria toxin inhibit protein synthesis in mouse Ehrlich-Lettre ascites carcinoma cells cultured *in vitro*. These observations could not be confirmed by us nor could the authors' further claim that toxin can cause regression of well-established ascites tumors in *preimmunized* mice be confirmed. Although temporary regression of such tumors can be demonstrated in unimmunized mice following intraperitoneal injection of diphtheria toxin, the amounts of toxin required are high and approach the lethal dose. About the same amount of CRM45, a *tox* gene product serologically related to diphtheria toxin but only 1/10,000th as toxic for guinea pigs, will also cause temporary regression in tumor-bearing mice.**

Since the discovery of diphtheria toxin more than 90 years ago, it has been known that mice and rats are exceptionally resistant to its lethal action (1). Similarly, cultured cells of mouse origin, such as L-cells, are unaffected by toxin concentrations several thousand times greater than are required to inhibit protein synthesis in cells from sensitive species (2–4). Recently, there have been reports that mouse Ehrlich ascites tumor cells carried in Swiss mice are more sensitive to the action of diphtheria toxin than are normal mouse cells either when tested *in vivo* (5, 6) or in cell culture (6). Indeed, Iglewski and Rittenberg (6) have claimed that protein synthesis in Ehrlich-Lettre (E-La) cells is at least 10,000 times more sensitive to inhibition by toxin than is protein synthesis in normal mouse cells. Largely, based on this finding, they have proposed that diphtheria toxin may prove a useful anti-cancer agent for human patients.

In the present paper we report our failure to confirm the experiments of Iglewski and Rittenberg (6). We will show that the apparent effect of toxin on E-La cells which they observed *in vitro*, can be explained most simply by their failure to supply factor(s) present in undialysed fetal calf serum that are needed for the maintenance of protein synthesis in these tumor cells.

While it appears to be true that diphtheria toxin can cause a striking, albeit temporary, regression of established Ehrlich ascites tumors, the doses required are large and very close to those that are lethal for mice. From our observations it seems unlikely that diphtheria toxin could be used effectively as a safe anti-cancer agent either in mice or in humans.

MATERIALS AND METHODS

Cells. Ehrlich-Lettre cells were kindly provided by Dr. C. E. Wenner of Roswell Park, Buffalo, N.Y. and were from the identical stock he had previously sent to Dr. Iglewski. The cells were maintained by weekly passage in Swiss-Webster mice. HeLa cells were a stock strain maintained in spinner culture on Eagle's minimal essential medium (MEM) containing 8% (antitoxin-free) horse serum.

Diphtheria Toxin and Serologically Related Proteins. Purified toxin was obtained from Connaught Laboratories, Toronto and was further purified by DE52 chromatography as previously described (7). The toxicity of the final product was approximately 25 guinea pig MLD per μg. CRM45 was isolated from culture filtrates of C7(β45) and purified as previously described (8). Fragment A was prepared from purified CRM30 that had been isolated from culture filtrates of C7(β30) (8). After brief treatment of CRM30 with trypsin in the presence of dithiothreitol, the fragment A so released was precipitated with ammonium sulfate and purified by DE52 chromatography. The final preparation was estimated to be about 70% pure both by sodium dodecylsulfate-gel electrophoresis and by measuring its ability to catalyse the transfer of [^{14}C]ADP-ribose from [^{14}C]NAD to inactivate Elongation Factor 2 (EF2), the eukaryotic polypeptidyl-tRNA-translocase (9).

Amino-Acid Incorporation. For experiments on protein synthesis, growing HeLa cells were centrifuged and resuspended to the desired cell density in MEM containing either 2 or 5% fetal calf serum (FCS, Grand Island Biologicals). Ascites tumor cells were collected from the peritoneal cavity of Swiss mice inoculated 6–7 days previously with about 3×10^7 E-La cells. About 4 ml of milky suspension containing about 2×10^8 cells per ml were collected per mouse. The cells were diluted with about 10 volumes of MEM, centrifuged, and washed twice more with the same volume of MEM. They were then diluted to the desired cell density in MEM containing 5% FCS. Protein synthesis was followed as a function of time in spinner flasks by measuring incorporation of [^{14}C]leucine as previously described (10) or, as in the procedure of Iglewski and Rittenberg (6), of ^{14}C-labeled amino-acid mixture (New England Nuclear, NEC445). In certain experiments, the medium was modified according to the procedure of Iglewski and Rittenberg (6) so as to contain 1% glutamine, 1/100th the usual concentration of other essential amino acids, and 2% FCS extensively dialysed against Hank's salt solution.

Mice. Female Swiss-Webster mice were obtained from Carworth Farms, Portage, Mich. and were used in most experiments. In certain experiments, the CD-1 Charles River Swiss strain was used.

RESULTS

Effect of diphtheria toxin on protein synthesis

Fig. 1A shows that [^{14}C]leucine incorporation into protein by Ehrlich-Lettre cells suspended in medium containing 5%

Abbreviations: E-La cells, Ehrlich-Lettre tumor cells; PBS, 0.02 M phosphate buffered saline (pH 7.2); MEM, minimal Eagle's medium; FCS, fetal calf serum; CRM, cross-reacting material; MLD, minimum lethal dose (in 250 g guinea pig); EF2, Elongation Factor 2; i.p., intraperitoneally.

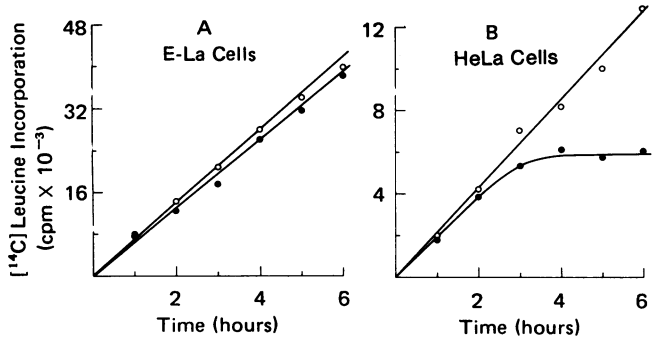

FIG. 1. Effect of diphtheria toxin on protein synthesis. (A) E-La ascites cells, (B) HeLa cells. In (A), ascites cells from mouse peritoneum were washed with MEM containing 5% FCS, suspended in 50 ml of MEM containing 5% FCS and 10 μCi [^{14}C]leucine, and then divided equally between two spinner flasks. To one flask was added 4 μg of toxin per ml; the other served as a control. The suspension contained 6.5 X 10^6 cells per ml. In (B), 3.3 X 10^5 HeLa cells per ml were suspended in the same medium in two spinner flasks, one of which contained 4 μg of toxin per ml. All four flasks were placed at 35° and duplicate 1 ml samples were collected on Millipore filters at hourly intervals, washed, and radioactivity counted in the usual manner. O—O, control; ●—●, toxin.

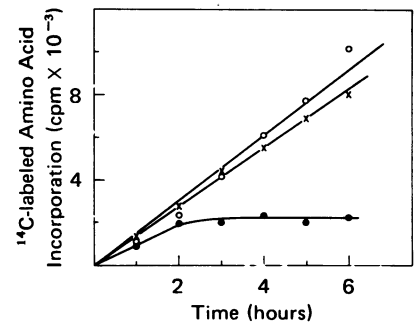

FIG. 2. Growth-stimulating properties of FCS before and after dialysis. Two spinner flasks, each containing 25 ml of MEM supplemented with 2% dialysed FCS (see *Materials and Methods*) and 10 μCi of ^{14}C-labeled amino-acid mixture were inoculated with 4.5 X 10^5 E-La cells per ml and 3 X 10^5 HeLa cells per ml, respectively. No toxin was added to either flask. Incorporation of label into protein was followed at 35° in the usual manner. ●—●, E-La cells; O—O, HeLa cells; x—x, [^{14}C]leucine incorporation by E-La cells (7 X 10^5/ml in MEM containing 5% *undialysed* FCS).

FCS was not reduced by 4 μg of toxin per ml over a 6 hr period. Fig. 1B shows that under identical conditions, the same concentration of toxin caused complete inhibition of leucine incorporation into HeLa cell protein within 3–4 hr.

Effect of dialysable serum factor on protein synthesis by E-La cells

In their assay for toxicity, Iglewski and Rittenberg (6) used cells that had been washed with MEM, modified so as to contain 1% glutamine but only 1/100th the normal concentration of the other amino acids. They suspended the washed cells in the same modified medium supplemented with 2% FCS that had been extensively dialysed against Hanks' balanced salt solution. After 3 hr incubation with various toxin dilutions at 37°, they added 1 μC ^{14}C-labeled amino-acid mixture per ml. The 3–5 hr incorporation into protein was then measured. Toxicity was expressed as percent cpm with toxin compared with a 2 hr pulse carried out with cells that had not been treated with toxin. From their paper, it is not clear whether or not their control cells without toxin had been incubated for 3 hr in the deficient medium before adding labeled amino acids.

Fig. 2 shows that, *in the absence of any added toxin*, when we used the normal concentration of amino acids and 2% *dialysed* FCS, protein synthesis in E-La cells began to slow down after 2 hr and amino-acid uptake had almost completely ceased after 3 hr at 35°. In the presence of 5% *undialysed* FCS, protein synthesis continued at the initial rate for at least 6 hr even at high cell densities as in Fig. 1A. It is obvious that toxicity cannot be meaningfully assayed by measuring amino-acid incorporation at a time when protein synthesis has already stopped for unrelated reasons. It is of interest to note that the S3 strain of HeLa cells does not require the factor present in FCS.

When a 3–5 hour pulse with ^{14}C-labeled amino-acid mixture was carried out *in the absence of toxin* using the modified medium described by Iglewski and Rittenberg (i.e., 1% the usual amino-acid concentrations except for glutamine, and 2% *dialysed* FCS) we found that incorporation of label into E-La cell protein was only about 20–25% of the uptake observed during the first 2 hr. Iglewski and Rittenberg (6) reported that incorporation of labeled amino acids during a 3–5 hr pulse was about 20% of the "control" value at all concentrations of toxin which they tested covering a 20,000-fold range from 0.003 to 60 μg/ml. They apparently did not test the effect of toxin concentrations below 0.003 μg/ml.

Effect of toxin and related proteins on established ascites tumors

Groups of four to six Swiss mice were injected intraperitoneally (i.p.) on day zero with E-La cells and 5 days later with diphtheria toxin. This is the same schedule described by Iglewski and Rittenberg (6). In other experiments, tumor-bearing mice were treated with CRM45 or with purified fragment A. It will be recalled that CRM45, the product of a mutant *tox* gene which lacks the 17,000 dalton C-terminal amino-acid sequence of toxin, is less than 1/10,000th as toxic for guinea pigs as toxin itself (11). Its A fragment is normal and has the same ADP-ribosylating activity on EF2 as fragment A isolated from toxin (11). Mice were weighed daily. About 3–4 days after injection of about 10^7 E-La cells, tumor-bearing mice began to gain weight at a high rate compared to uninjected control mice. In Fig. 3, the average weight gain of each group is expressed as percent of that group's average weight on day zero. A decline in rate of weight gain was taken to indicate tumor regression. Fig. 3 shows that according to this criterion, a single dose of 2.5 μg or more of either toxin itself or of CRM45 caused regression of ascites tumors to begin within 48 hr. The treated animals began to lose weight 4–5 days after injection of either toxin or CRM45 and at the same time most of them began to show signs of paralysis. Most animals died of intoxication within less than 9 days. In one instance, a mouse that had received 4.5 μg of toxin was autopsied 1 hr after death on the fourth day. Although marked regression of the tumor had occurred, a cell suspension could be obtained by washing out the peritoneal cavity with MEM. This suspension was injected into three mice, all of which developed tumors within 6 days. Some of the tumor-bearing mice that had been treated with a single dose of about 2.5 μg of toxin and had shown weight loss, recovered from the effects of toxin, only to have tumor growth resume once more. Tumor-bearing mice that had received 10 units of rabbit or of horse antitoxin 24 hr before injection of either intact toxin or CRM45, continued to gain weight at a rapid rate and tumor development continued unchecked. Purified fragment A was less effective as an

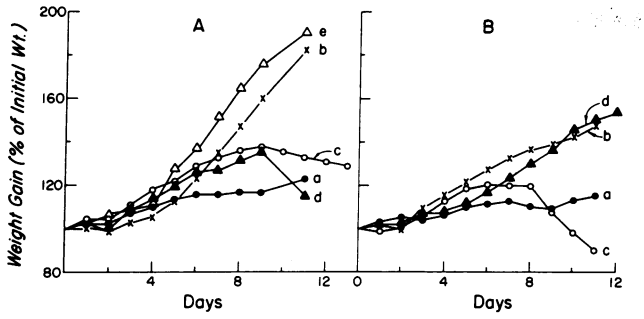

FIG. 3. Growth of Swiss mice following injection of E-La cells. In (A), groups of five to six mice, averaging 18–19 g, received 2×10^7 E-La cells intraperitoneally (i.p.) in a volume of 0.2 ml on day zero. Four days later, mice in *group b* each received 10 units rabbit diphtheria antitoxin, i.p. On the fifth day, mice in *groups b* and *d* were injected with 4 μg of toxin in 0.5 ml of PBS; *group c* received 2.5 μg of toxin. *Group a* were uninjected normal controls; *group e* received tumor cells but no toxin. Mice were weighed daily and the weights averaged for each group. All mice in *group d* showed signs of paralysis 5 days after injection with toxin. One mouse in this group died on the sixth day. In *group c* (2.5 μg of toxin), all mice showed signs of paralysis within 6 days; they were sacrificed 8 days after injection of toxin. In (B), groups of mice averaging 25 g in weight were injected with 6×10^6 E-La cells on day zero. *Group b* received 10 units of horse antitoxin on day 3. On day 4, mice in *group c* were injected with 3.5 μg of CRM45 and those of *group b* with 7 μg of CRM45. *Group d* received tumor cells only. *Group a* were uninjected normal controls. All mice in *group c* showed signs of paralysis by the fifth day after injection of CRM45. One mouse in this group died on day 7; the others were in poor shape and were sacrificed. In a separate experiment, four mice injected with 7 μg of CRM45 all died on the sixth day.

anti-tumor agent, although some temporary regression and loss of weight occurred in mice that received multiple injections of several μg each.

DISCUSSION

The present studies clearly show that fetal calf serum contains a factor which is necessary for growth of freshly isolated Ehrlich ascites tumor cells (Lettre strain). The factor is not required by the S3 strain of HeLa cells. This is not a novel observation since the importance of serum factors in the cultivation of animal cells has been demonstrated repeatedly (12). We find that although amino-acid incorporation into protein by washed suspensions of E-La cells in MEM supplemented with *undialysed* FCS will continue at an undiminished rate for many hours, incorporation ceases after 2–3 hr when 2% *dialysed* FCS is used as a supplement. It is obvious that under such conditions, incorporation of a labeled amino-acid mixture into protein during a 3–5 hr pulse has very little meaning and cannot be used to measure sensitivity to a bacterial toxin as claimed by Iglewski and Rittenberg (6). As shown in Fig. 1, even high concentrations of toxin have no effect on protein synthesis by E-La cells over a 6 hr period in the presence of undialysed FCS.

The relatively high resistance of mice and rats and of cultured cells from these species to diphtheria toxin is apparently due to a lack of membrane receptors specific for the toxin B-polypeptide chain (fragment B). Such receptors are present on cell membranes of toxin-sensitive species and have been shown to facilitate entry of the A-polypeptide to reach the cytosol where it arrests protein synthesis by catalyzing the NAD-mediated inactivation of EF2 by ADP-ribosylation. Evidence for the existence of specific membrane receptors comes from studies on binding of ^{125}I-labeled toxin to cultured human and mouse cell lines (ref. 13 and unpublished work of P. Boquet and A. M. Pappenheimer, Jr.), from the isolation of toxin-resistant human cell lines with altered surface properties (14) and finally, from studies on the sensitivity to toxin of hybrid cells formed by fusion of resistant mouse cells with sensitive chicken (15), human (16) or monkey (17) cells. The studies with hybrid cells have shown that a gene controlling sensitivity to toxin is absent from mouse cells. When a hybrid cell loses the particular chromosome that carries the toxin-sensitivity gene, the hybrid becomes resistant once more (16, 17).

There have been several reports in the literature that sublethal injections of certain toxic proteins will cause regression of Ehrlich ascites tumors in mice. Lin and his associates (18) showed that the toxic seed proteins, abrin and ricin, almost completely suppress growth of ascites tumors if given to mice in two properly spaced sub-lethal doses within the first 48 hr after injection of the tumor cells. The amount of toxin required for regression and prolonged survival time varied somewhat with different strains of mice but was always close to the lethal dose. Analogous findings were reported by Buzzi and Maistrello (5) who obtained regression of Ehrlich ascites tumors and prolonged survival of tumor-bearing mice following multiple injections of sub-lethal doses of diphtheria toxin. Treatment with toxin had little effect if begun after the third day. Although Buzzi and Maistrello used a crude culture filtrate as the source of their toxin, they found no anti-tumor effect using toxin that had been preincubated with an equivalent amount of specific diphtheria antitoxin. In the experiments of Iglewski and Rittenberg, however, mice received three injections of diphtheria toxoid in complete Freund adjuvant prior to i.p. injection of 5×10^7 E-La cells. Five days later each mouse received 3 μg of toxin. In the latter experiments, no attempt seems to have been made to determine whether or not the immunized mice had actually produced antitoxin. At any rate, despite the fact that they injected 50 times as many tumor cells as Buzzi and Maistrello (5) into mice that had been *preimmunized*, and then waited 5 days before treatment with a *single* dose of toxin, Iglewski and Rittenberg (6) obtained regression of their tumors.

In our own experiments, tumors failed to regress in mice that had received antitoxin 24 hr prior to treatment with toxin.

Although mice are several thousand times more resistant to the lethal action of diphtheria toxin as are guinea pigs or humans, they do develop paralysis and die after receiving doses of 3 μg or more. With such high doses, enough toxin probably becomes internalized by a nonspecific endocytotic mechanism. Probably, a few molecules of the relatively resistant A-fragment manage to escape degradation by lysosomal enzymes and reach the cytoplasm to block protein synthesis in a sufficient number of cells to cause death of the animal. Such a nonspecific endocytotic mechanism of entry would explain why CRM45 is just as toxic for mice and just as effective in causing regression of mouse ascites tumors as is toxin itself. Yet CRM45 is less than 1/10,000th as toxic as intact toxin when injected into guinea pigs. Ascites cells are actively pinocytotic. It may well be that the increased sensitivity to toxic proteins reported for ascites tumor cells and even solid tumors may be attributed to their high pinocytotic activity and to the fact that the toxin is injected directly into the tumor. Such a mechanism would also explain the results of Buzzi and Maistrello (5) who used a crude toxin with only 7–8 guinea pig MLD/Lf (equivalent to only 2–3 MLD

per μg as compared with about 25 MLD per μg in the present experiments). From the work of Gill and Dinius (19), preparations of such low specific toxicity almost certainly contained degraded toxin which nonetheless still flocculated with antitoxin. Such degraded material would be expected to behave similar to CRM's such as CRM45.

In conclusion, we can find no convincing evidence, either from our own experiments or those of others, that Ehrlich ascites tumor cells are any more sensitive to the action of diphtheria toxin than are the cells of normal mice. Any suggestion that diphtheria toxin may prove to be a useful agent in the treatment of human cancer would appear to be premature at this time.

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