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STUDIES OF THE BIOLOGIC ACTIVITY OF PURIFIED STAPHYLOCOCCAL ALPHA-TOXIN: II. THE EFFECT OF ALPHA-TOXIN ON EHRLICH ASCITES CARCINOMA CELLS†

Previous studies[1] have indicated that highly purified staphylococcal α-toxin damages a variety of cells growing in culture. Rabbit kidney tissue appears to be especially susceptible, and undergoes not only gross morphologic changes but also injury to cellular membranes resulting in the release of intracellular substances of high molecular weight. The purpose of the present paper is to report some observations on the effects of staphylococcal α-toxin of varying degrees of purity on the morphology, permeability and invasiveness of Ehrlich ascites carcinoma (EAC).

Living streptococci and streptococcal toxins have been found to be markedly cytotoxic for EAC and to decrease the invasive capacity of the tumor cells for mice.[2-4] These effects were not found with live staphylococci.[3] Ginsburg and Grossowicz[3] have suggested that the mechanism involved in this phenomenon is probably cell membrane damage resulting in alteration of permeability and leakage of intracellular material.

MATERIALS AND METHODS

The staphylococcal α-toxin and antitoxin used in these studies were similar to those described earlier[1] and satisfied the established criteria for purity, lethal and dermonecrotic activities, and freedom from detectable beta and delta hemolytic activity.[5, 6] Toxin was inactivated by heating at 60° C. for 30 minutes.

Animals. Male Swiss white mice, 20 to 24 grams in weight, were employed for all the experiments.

Cells. The strain of Ehrlich ascites carcinoma‡ (EAC) was maintained by intraperitoneal injection of 0.2 ml. of freshly obtained ascitic fluid into mice every 6 days.

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Peritoneal fluid was removed by needle aspiration 7 to 10 days after the animals were inoculated, centrifuged at about 400 g., washed three times with Hanks' solution and resuspended to the initial concentration in Eagle's basal medium to which 2 per cent calf serum was added. The cell mass comprised 15 to 30 per cent of the total volume and was free of erythrocytes.

Radioisotope studies. Tumor cell suspensions were prepared, as described above, in Eagle's medium free of methionine plus 2 per cent calf serum. S^{35}-L-methionine* was added to a final concentration of 5 μg./ml. and the mixture incubated at 37° C. in a rotary water bath (60 strokes per minute) for 4 hours. The cells were then washed several times and suspended in Hanks' solution. Unheated or heated α-toxin made isotonic with 10x concentrated medium 199 was added to identical aliquots of cell suspensions at the beginning of each experiment and samples removed at zero time and at specific intervals thereafter. All ingredients were warmed to 37° C. immediately before each study. Toxin activity was halted either by the addition of antiserum or, in some experiments, by putting the material into tubes in an ice bath. The cells were removed immediately from suspension by centrifugation in the cold and the supernatant fluid examined for radioactivity. Direct counting was carried out by plating at infinite thinness on stainless steel planchets. The methods for determination of radioactivity in cells and protein were described previously.[1]

RESULTS

VISIBLE EFFECTS OF ALPHA-TOXIN ON EHRLICH ASCITES CARCINOMA CELLS IN VITRO

Striking morphological changes appeared in the EAC cells within a few minutes of exposure to staphylococcal α-toxin (Fig. 1). The nucleus became prominent and intranuclear bodies and cytoplasmic granules appeared. Balloon-like extrusions developed and persisted. Staining with trypan blue revealed an increasing death rate until 30 minutes after exposure to toxin, when all cells were stained. Only 5 to 10 per cent of cells that were untreated or in contact with heat-inactivated toxin died. The packed volumes of suspensions exposed to toxin were found to be twice as large as those to which heated toxin had been added, indicating that the "ballooning" process was associated with an increase in cell size. Purified and crude toxin preparations produced the same results. That the morphologic alterations which developed were in part dependent on the quantity of toxin was suggested by the fact that highly concentrated material produced cell enlargement, nuclear prominence and death beginning within a few seconds.

EFFECT OF TOXIN ON TUMOR PROLIFERATION IN MICE

Effect of in vitro treatment of Ehrlich ascites carcinoma with α-toxin. Ehrlich ascites carcinoma cells which had undergone the changes described

* Obtained from Radiochemical Centre, Amersham, England.

above, when exposed to staphylococcal α-toxin *in vitro,* failed to proliferate after intraperitoneal injection into mice.

In preliminary experiments, partially purified toxin (post-sephadex or post-electrophoresis[5] — hemolytic titer of 640 units per ml. for rabbit erythrocytes) was mixed with an equal volume of suspension of Ehrlich ascites carcinoma cells (10^8 per ml.) and incubated at 37°C. for one hour. One volume of a 1 : 40 dilution of antitoxin was then added, and the mixtures allowed to stand at room temperature for 15 minutes. Tumor cells exposed

TABLE 1. PATHOGENICITY OF EHRLICH ASCITES CARCINOMA (EAC) INCUBATED WITH PARTIALLY PURIFIED STAPHYLOCOCCAL α-TOXIN FOR ONE HOUR

Materal injected	*No. of mice*	*No. with ascites after 6-15 days*	*No. of deaths 15th day*
E.P. toxin* + EAC + antitoxin**	3	0	0
G-75 toxin* + EAC + antitoxin	3	0	0
G-75 toxin (heated) + EAC + antitoxin	3	3	3
G-75 toxin + EAC + Hanks' solution	3	..†	3†
EAC + Hanks' solution	3	3	3
E.P. toxin + Hanks' solution	3	..†	3†

* Reference 5.
** Antitoxin added at end of incubation period, mixtures held at room temperature for 15 minutes prior to intraperitoneal injection of 0.6 ml.
† All dead within 24 hours.

to heated toxin and antiserum, or untreated, served as controls. Six-tenths of a milliliter of each mixture was injected intraperitoneally into each of three mice. The animals given control cell suspensions all developed ascites within 6 days and died in 15 days or less. Those receiving cells which had been in contact with α-toxin subsequently neutralized by antitoxin showed no evidence of accumulation of peritoneal fluid and survived. When mice were inoculated with Ehrlich ascites carcinoma treated with unneutralized α-toxin, they succumbed within 24 hours, probably as a result of the activity of the staphylococcal product (Table 1).

The effect of the duration of exposure of Ehrlich ascites carcinoma to α-toxin *in vitro* on its ability to proliferate in mice was determined in the following manner: A suspension of tumor cells was incubated with purified α-toxin (300 hemolytic units/ml.) at 37° C. in a rotary shaker bath and aliquots removed after 1, 2, 5, 10, 30 and 60 minutes. Six-tenths of a milliliter of each preparation was injected intraperitoneally into each of 6 mice. The results of a typical experiment are presented in Table 2. Preincubation of toxin and cells for 5 minutes or less did not appear significantly

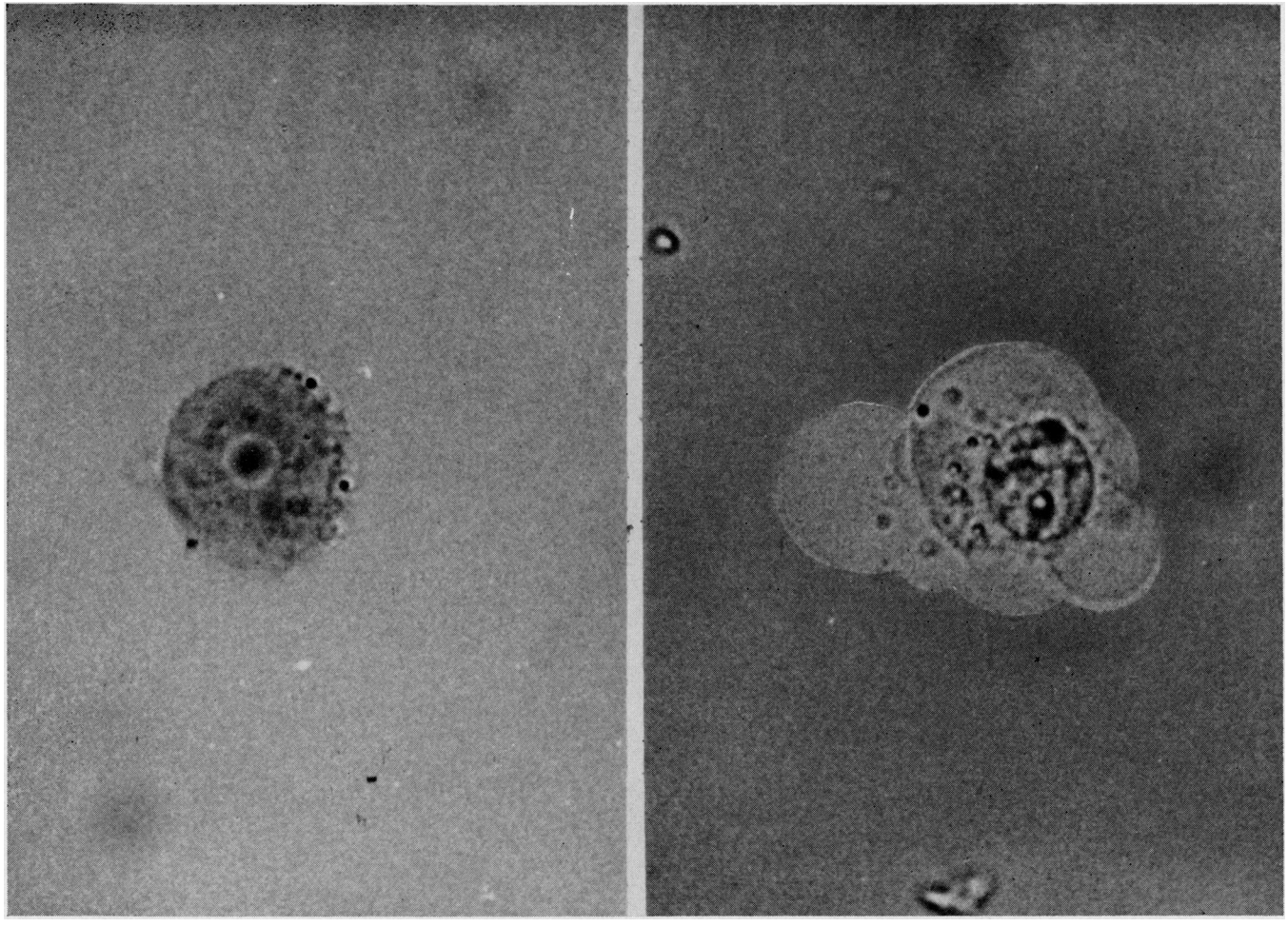

FIG. 1 (left). Normal appearance of Ehrlich ascites carcinoma cell.
FIG. 1 (right). Ehrlich ascites carcinoma cell, 10 minutes after exposure to purified staphylococcal α-toxin. Nucleus prominent. Multiple cytoplasmic extrusions clearly visible.

to alter the development of ascites or the percentage of surviving animals. Contact of the tumor with toxin for 10 to 30 minutes decreased but did not abolish its capacity to multiply intraperitoneally and to produce death, since all of the animals finally succumbed. Cells treated with purified toxin for 60 minutes still produced ascites and death in one of the 6 animals.

TABLE 2. PATHOGENICITY OF EHRLICH ASCITES CARCINOMA EXPOSED TO PURIFIED STAPHYLOCOCCAL α-TOXIN FOR VARYING TIME PERIODS*

Time	No. of mice	No. with ascites Day 6-9	10-12	13-15	16-25	No. dead Day 12	14	16	18	22
1 minutes	6	6	..	..	..	0	3	3	4	6
2 minutes	6	6	..	..	..	2	4	5	6	6
5 minutes	6	6	..	..	..	2	4	6	6	6
10 minutes	6	0	6	..	..	0	0	1	3	6
30 minutes	6	0	0	2	6	1	1	1	2	6
60 minutes	6	0	0	0	1	0	0	0	0	1
Control† 2 minutes	6	6	..	..	..	3	5	6	6	6
Control† 10 minutes	6	6	..	..	..	4	5	6	6	6
Control† 60 minutes	6	6	..	..	..	4	5	6	6	6

* Cell-toxin suspensions held at room temperature with antitoxin for 10 minutes prior to intraperitoneal injection.
† Heated toxin (60° C. for 30 minutes).

Effect of α-toxin on Ehrlich ascites carcinoma in vivo. The effect of purified α-toxin on Ehrlich ascites carcinoma cells growing in the peritoneal cavity of animals was investigated. Several groups of mice were inoculated intraperitoneally with 0.2 ml. of a suspension of tumor cells. Four days later, 0.2 ml. of toxin was injected. Antitoxin was given to one group after 1 minute, to a second after 10 minutes, to a third after 60 minutes, and to a fourth after 120 minutes. Control animals received toxin or antitoxin alone or sterile buffer.

The mice which did not receive antitoxin died within 24 hours. Those which were given toxin plus antitoxin, buffer or antitoxin alone developed ascites within 3 days and died within 16 days of the implantation of the tumor. Animals injected with antitoxin subcutaneously 2 hours prior to the intraperitoneal instillation of toxin succumbed within one day. When the antitoxin was administered under the skin 18 hours before the toxin, death was delayed to the same degree as that observed when all injections were made into the peritoneal cavity.

The effect of repeated exposure of Ehrlich ascites carcinoma growing in

the peritoneal cavity of mice to purified toxin was also studied. The disease was established in the manner described above. On the fourth day after inoculation of the tumor, 3 groups of animals were given antitoxin subcutaneously; in one, toxin was administered intraperitoneally on the 5th day, in the second on the 5th and 6th days, and in the third on the 5th, 6th and 7th days. Several groups of mice were given antitoxin subcutaneously daily for 2 to 6 days, followed, at 24 hour intervals, by toxin.

Animals which received toxin in excess of antitoxin succumbed within 48 hours from the effects of the toxin. The rest of the toxin-treated passively immunized animals developed carcinomas and died at the same rate as the controls.

The results of these studies indicated that α-toxin, although able to destroy the proliferative capacity of Ehrlich ascites carcinoma cells exposed *in vitro,* did not alter the course of the disease when it was produced in animals. Since it was necessary to administer antitoxin in order to protect the mice against the lethal effects of the toxin, the possibility that neutralization of the toxin prevented it from acting on the carcinoma *in vivo* must be considered. Antibodies appear in high concentration in the ascitic fluid of mice with Ehrlich ascites carcinoma.[7] Because solid tumor masses may develop on the peritoneal surface and in various abdominal organs, it is possible that most of the cells were inaccessible to the toxin. In support of this possibility is the fact that toxin administered as long as 2 hours before antitoxin did not affect the course of the disease.

RELEASE OF INTRACELLULAR SUBSTANCES FROM EAC BY ALPHA-TOXIN

The results of studies of the release of intracellular substances from Ehrlich ascites carcinoma cells exposed to α-toxin supported the previously-proposed hypothesis[1] that this substance produces direct damage to structural components of the cell membrane, resulting in alteration and disruption of the permeability characteristics of this barrier.

Two aliquots were removed from a suspension of Ehrlich ascites carcinoma cells labelled with S^{35}-L-methionine, prepared as described above. One portion was mixed with an equal volume of purified α-toxin made isotonic with 10x concentrated medium 199 and the other with isotonic heat-inactivated toxin. All of the fluids were prewarmed to 37°C., and then incubated at this temperature. Samples were removed from each mixture at 0, 2, 5, 15, 45, and 90 minutes and added to an equal quantity of dilute horse antiserum (1:40) or, in some experiments, placed in chilled tubes. The cells and supernatant fluid were rapidly separated by cold contrifugation for determination of S^{35}-activity.

The release of S^{35}-L-methionine from cells exposed to purified α-toxin was rapid; peak total values were reached in 5 minutes and maintained at a fairly constant level thereafter. Study of controls revealed an initial sharp rise which was slower than that observed with the treated cells and which gradually reached a maximum after about 45 minutes (Fig. 2). This probably reflected the establishment of equilibrium of the S^{35}-L-methionine in the intracellular pool with the methionine-free extracellular medium.

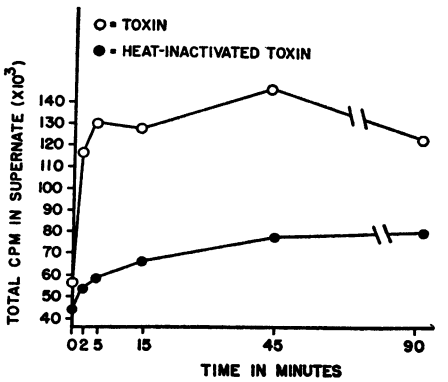

FIG. 2. The Release of S^{35}-L-methionine from Ehrlich ascites carcinoma cells exposed to purified staphylococcal α-toxin.

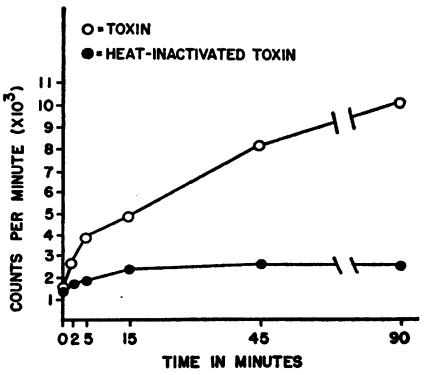

FIG. 3. The release of TCA-precipitable S^{35}-L-methionine from Ehrlich ascites carcinoma cells exposed to purified staphylococcal α-toxin.

Determination of TCA-precipitable S^{35}-L-methionine also revealed an initial sudden release, comparable to that observed when total S^{35}-L-methionine was measured, and then a gradual, nearly linear increase through the remainder of the period of study (Fig. 3). A maximum of 8.7 per cent of the methionine released from the cells was in a TCA-precipitable state; this was in sharp contrast to the 70 per cent release from rabbit kidney cells.[1] Strauss and Hendee[8] found virtually all methionine incorporated into growing HeLa cells in protein. These findings are similar to those made by us in rabbit kidney and human amnion tissue cultures.[1] However, in tumor cells incubated with S^{35}-L-methionine, maximum incorporation (17 per cent) occurred in 4 hours. This suggested that, under the conditions of the experiment, the bulk of methionine was in the free form in the intracellular pool, and that only a relatively small quantity was bound, by incorporation or adsorption, to protein.

The results of these studies suggest that damage to cell membrane by α-toxin is prompt. The physical changes which appear increase in severity

with duration of exposure. The initial event is probably rapid loss of substances of small molecular size; this is followed by the gradual sustained release of larger compounds, as disruption of the membrane progresses.

DISCUSSION

The data presented in this and the preceeding paper[1] suggest that the primary action of staphylococcal α-toxin on cells is direct damage to their membrane, which leads to loss of cellular integrity and culminates in lysis. The specific biochemical site which is affected is not known. It is highly unlikely, however, that death and disintegration of the cell result from interference with intermediary metabolic processes such as inhibition of nucleic acid or protein synthesis. The extreme rapidity with which the toxin acts points strongly to a direct attack on membrane structural components; this appears also to be the case with several other cell lysins of microbial origin.[9]

The extent to which the effects of α-toxin on free tumor cells and tissue cultures are reversible is presently being investigated in this laboratory. Studies of the kinetics of the activity of purified α-toxin and rabbit erythrocytes have demonstrated a pre-lytic lag phase, in the early part of which the addition of antiserum prevents the release of hemoglobin. Whether a similar phenomenon occurs with substances of lower molecular weight is not yet known. Since Ehrlich ascites carcinoma cells are known to concentrate free amino acids to a great degree,[10] the relatively poor incorporation of S^{35}-L-methionine into the protein of the tumor, in contrast to that which occurs in HeLa and rabbit kidney cells, is not surprising.

It has also been pointed out that these cells are relatively more permeable to intracellular substances than are other types of tissues.[11] The comparatively rapid release of non-TCA precipitable S^{35}-L-methionine suggests a gradual increase in the degree of membrane damage.

Ginsburg and Grossowicz[3] have demonstrated similar morphologic alterations and effects on the proliferative capacity of Ehrlich ascites tumor cells incubated with streptococcal hemolysins. With the exception of streptolysin 0, the appearance of damage was delayed for several minutes, suggesting that the changes, although qualitatively similar to those produced by staphylococcal α-toxin, were due to a different mechanism than that involved in the activity of the latter. Such differences might, however, be only dose-related.

It should be emphasized that both crude and highly purified staphylococcal α-toxin appear to produce the same cell-damaging effects. This suggests that the observed anatomical and biochemical injuries are probably induced by

the activity of a single substance which is most likely enzymatic in nature. Kumar and associates[12] have reported that a purified α-toxin having electrophoretic properties identical, for the most part, with the preparation used in the present study produced hemolytic, dermonecrotic, lethal and leucocidic effects. This lends support to the concept that staphylococcal α-toxin has a broad range of cellular activity.

SUMMARY

Ehrlich ascites carcinoma cells exposed *in vitro* to either crude or highly purified staphylococcal α-toxin showed pronounced morphologic changes and failed to proliferate in mice. There was no demonstrable effect on established tumors in mice passively immunized against the lethal effects of the toxin. Alpha-toxin permitted a marked increase in the release of trichloroacetic acid-precipitable S^{35}-L-methionine following the rapid release of free amino acid from Ehrlich cells. It is proposed that α-toxin acts by altering the permeability characteristics of the cell membrane.

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