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STUDIES OF THE BIOLOGIC ACTIVITY OF PURIFIED STAPHYLOCOCCAL ALPHA-TOXIN: I. THE EFFECT OF ALPHA-TOXIN ON CELLS IN TISSUE CULTURE†

Recent studies with highly purified preparations of α-toxin[1-3] have supported the concept that the hemolytic, dermonecrotic, leucocidic and lethal effects result from the activity of a single substance. Although there is general agreement that the erythrocytes of various animal species exhibit different degrees of susceptibility to hemolysis by α-toxin,[4] the mechanisms which account for this variability are unknown.

The selective action of the alpha-toxin of *Staphylococcus pyogenes* (var. *aureus*) on cells and tissues of different animals has been the subject of considerable controversy.[4] Several investigators[5-9] have examined the effects of this material on a number of cell types in culture. In most instances, cytotoxic effects, qualitatively similar regardless of the origin and nature of the tissues, have been demonstrated; variation in the speed and intensity of the reactions has been noted, however. Thal and Egner[8] have emphasized the special sensitivity of smooth muscle cells to the action of α-toxin.

Although the biochemical site of α-toxin action is unknown, the rapidity with which it produces cell changes suggests that it probably does not inhibit intermediary steps in protein synthesis, as does diphtheria toxin,[10-12] but that it may injure cell membranes.

The purpose of this and the succeeding paper[13] is to present the results of studies of the qualitative and quantitative aspects of the activity of highly purified staphylococcal α-toxin on cells derived from various sources.

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† This work was carried out with support from the United States Public Health Service grant E-2564(C3).

Received for publication 8 October 1962.

MATERIALS AND METHODS

Staphylococcal α-toxin. Highly purified staphylococcal α-toxin was prepared and assayed by the methods previously described.[3] Unless otherwise indicated, the material yielded a single band when subjected to agar double diffusion against several dilutions of potent horse antiserum* and possessed no detectable beta or delta hemolytic activity.[4]

Cell cultures. Human amnion tissue cultures (HATC) were prepared by trypsin treatment of sterile amnion.** One milliliter of the suspension containing 400,000 cells per milliliter were placed in round screw-cap tubes. Leighton tubes were inoculated with twice this volume. The medium used for growing the tissue cultures was Eagle's to which 15 per cent heat-inactivated horse serum was added. Eagle's medium plus 2 per cent heated calf serum served as the maintenance fluid.

Rabbit kidney cell cultures (RKTC) were prepared by inoculation of trypsinized minced tissue of animals, ranging in age from 2 weeks to several months, into a medium consisting of Hanks' balanced salt solution, lactalbumin hydrolysate (0.5 per cent final concentration) and 4 per cent heated calf serum. The cells were maintained in Eagle's basal medium containing 2 per cent calf serum. Mouse kidney cultures were prepared in similar fashion. Swiss mice less than 2 weeks of age were used. Rhesus monkey kidney cells were obtained from Microbiological Associates, Bethesda, Maryland.

Virus. The *herpes simplex* virus used in these studies was a primary isolate maintained by serial passage in human amnion tissue culture. Influenza virus (PR8) was grown in the allantoic sac of 10 day old embryonated hen eggs.

Radioisotope studies. Cultures of all the tissues studied were incubated at 36°C. for 24 to 78 hours with 1 ml. of Eagle's basal medium (free of nonradioactive methionine) to which S^{35}-L-methionine*** was added in a concentration of 5μg. per ml. Each culture was washed eight times with 1.5 ml. of cold Hanks' solution before incubation with heated (60°C. for 30 minutes) or unheated toxin made isotonic with 10X-concentrated medium 199.

The supernatant fluids from 10 tubes in each group were pooled at specific intervals and centrifuged at 2,500 r.p.m. for 10 minutes at 4°C. The supernates were recentrifuged and aliquots of each plated at infinite thinness on stainless steel planchets for direct counting of radioactivity.

For estimation of the degree of protein release from cells treated with heated and unheated toxin, representative aliquots of tissue culture fluids were mixed with an equal volume of cold 10 per cent trichloracetic acid (TCA) and allowed to stand at 4° C. for 16 hours. The precipitates were collected on Millipore filters of 0.45 mμ pore size, washed several times with chilled 5 per cent TCA containing 150 μg. of nonradioactive methionine per milliliter and then mounted on steel discs and dried.

Determinations of the S^{35}-L-methionine in protein by centrifugation of the TCA precipitates and solution in 6N NH_4OH prior to plating and counting on steel planchets yielded results nearly identical with those obtained by the Millipore filter method.

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* Kindly supplied by Dr. Mollie Barr, Wellcome Research Laboratories.

** Obtained through the courtesy of Dr. W. A. Bardawil, St. Margaret's Hospital, Boston, Massachusetts.

*** Obtained from Radiochemical Centre, Amersham, England.

The quantity of S^{35}-L-methionine in cells was determined in the following manner: Aliquots of cell suspensions were removed from the glass tube with ethylenediaminetetracetic acid, collected on Millipore filters and washed with cold Hanks' solution. For measurement of the degree of incorporation of the isotope into cell protein, these were then washed with cold 5 per cent TCA containing 150 micrograms of nonradioactive methionine per milliliter.

Counting of radioactivity was carried out in a thin window gas-flow counter (Baird Atomic). Duplicate determinations were found to agree in the range of accuracy of ± 5 per cent.

RESULTS

CYTOPATHIC CHANGES IN TISSUE CULTURES

Rabbit kidney. Alterations in the appearance of monolayers of kidney cells on glass slides appeared within minutes after exposure to staphylococcal α-toxin (Fig. 1a). Balloon-like processes extruding from the cytoplasm were visible in 3 minutes (Fig. 1b). Tube cultures examined under low magnification revealed the first cellular changes in about 10 minutes. At this time, the cells began to separate from each other and new semicircular extrusions appeared. The architecture of the monolayer then became disrupted as the cells separated from the glass. When studied by phase contrast microscopy, the "balloon" processes were noted to detach after 25 minutes and to float in the fluid covering the tissue.

Shortly after "ballooning" appeared, the unstained nucleus, normally seen indistinctly, became lighter in appearance and its membrane thickened and became darker. As the process progressed, the nucleus decreased in size and was accentuated by the formation of a clear area around it (Fig. 1c and d). Granularity of the cytoplasm developed in the late stages. The final event was virtually complete separation of the cells from the glass.

Human amnion. The first changes produced by staphylococcal α-toxin in human amnion occurred in the cell nuclei. These decreased in size and became more easily visible and refractile in unstained preparations. As the nuclear membrane thickened, a clear space appeared between it and the cytoplasm. The usual homogeneous appearance of the nucleus was altered so that it became more transparent; the nucleoli darkened and stood out in sharp contrast. A number of different preparations of toxin of varying degrees of purification produced similar cellular effects (Figure 2a and b).

Extrusion of cytoplasmic material, as noted in rabbit kidney cells, was observed only once in human amnion, by phase microscopy after exposure to a purified toxin preparation (hemolytic titer-1:600). Human amnion cells failed to separate from the glass even several days after they were killed; this differed from the reaction of rabbit kidney cells exposed to

toxin. Cell death was indicated by uptake of trypan blue, absence of pH change of the maintenance medium to an acid reaction and inability to become infected with *herpes simplex* virus. Amnion cell suspensions to which α-toxin was added prior to distribution in tubes did not become attached to the glass surface.

Cytopathic effects similar to those produced in rabbit kidney by α-toxin were also observed in mouse and monkey kidney cell cultures grown in round tubes. The CPE produced in the several tissue culture types by crude and partially purified toxin appeared identical with that caused by highly purified material.

MECHANISM OF ACTION OF α-TOXIN ON CELLS IN TISSUE CULTURE

The rapidity with which evidence of tissue damage developed after exposure to staphylococcal α-toxin suggested the possibility that the

TABLE 1. EFFECT OF STAPHYLOCOCCAL α-TOXIN ON INFECTION OF HUMAN AMNION BY HERPES SIMPLEX VIRUS

	Dilution of virus causing CPE	
Experiment	*Toxin*	*Heated toxin*
1	$10^{-2.5}$*	$10^{-2.5}$
2	$10^{-2.5}$	$10^{-3.0}$

* Virus titer per 0.1 ml. of virus-toxin-antitoxin mixture.

primary site of injury was the cell membrane. Experiments were carried out to examine this hypothesis.

Release of virus from infected cells. Fluids for titration of virus infectivity were clarified by centrifugation. Equal volumes of purified toxin (640 hemolytic units per milliliter) and virus fluid were mixed and incubated at 37° C. for one hour. One volume of a 1:40 dilution of antitoxin (800 units per milliliter) was then added to neutralize the toxin. Serial tenfold dilutions of this mixture were made in balanced salt solution and 0.1 ml. of each dilution inoculated into each of two human amnion cell culture tubes. Controls consisted of virus fluid exposed to heated toxin and studied in a similar manner. The virus titer was considered to be the highest dilution of the virus-toxin-antitoxin mixture which produced viral cytopathic effect after 3 to 5 days of incubation at 37°C. Differences of less than one logarithm are not usually considered significant by this technique. As indicated in Table 1, purified α-toxin did not have a deleterious effect on *herpes simplex* virus.

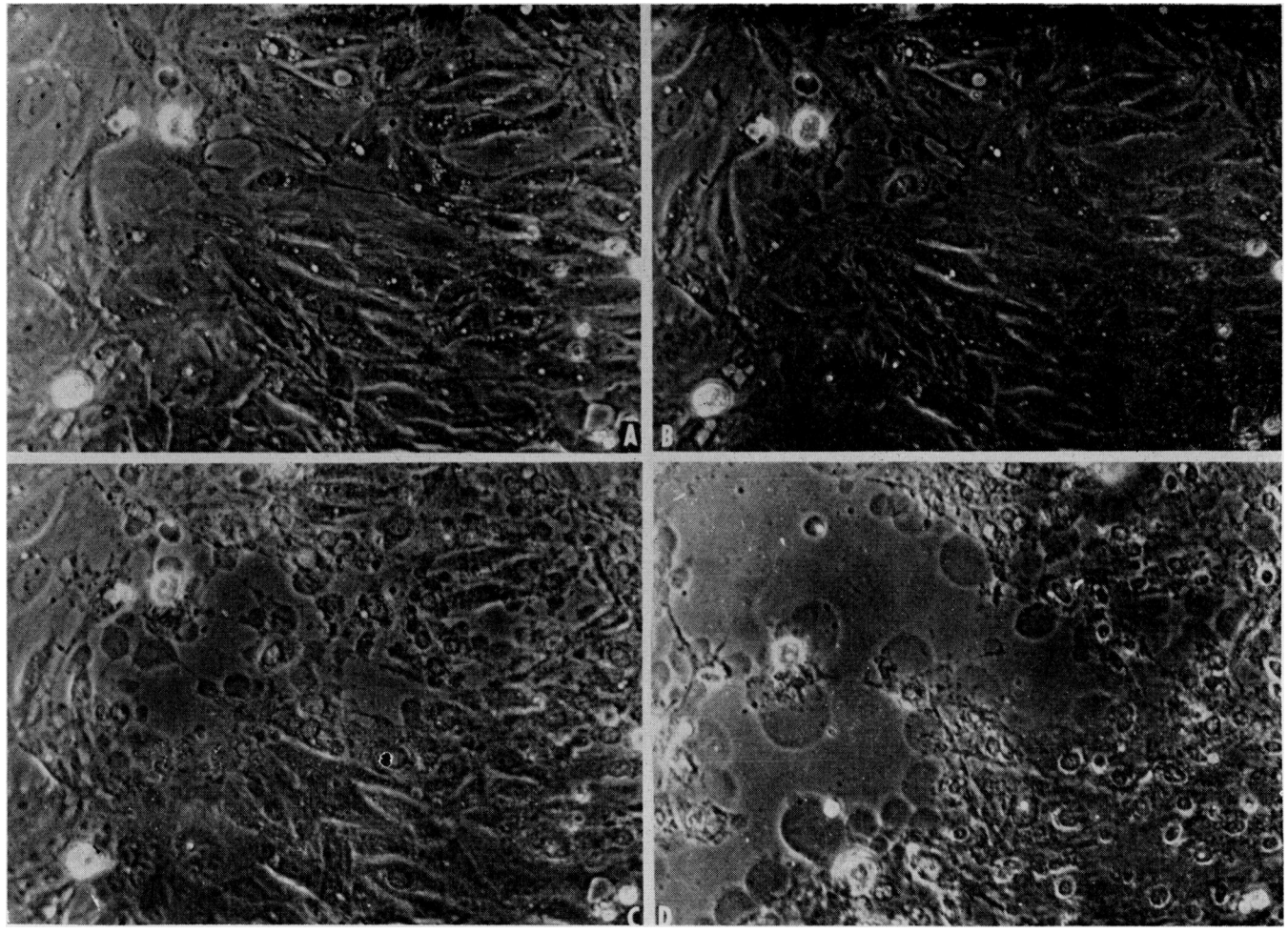

FIG. 1. Rabbit kidney tissue culture after addition of purified staphylococcal α-toxin. Glass cover-slip preparation. Single field. Phase-contrast microscopy. 200x magnification.

a. One minute: Normal appearance of monolayer.
b. Three minutes: Early "ballooning" of cytoplasm and separation of cells in one area.
c. Seven minutes: Cellular extrusions more extensive. Nuclear changes prominent.
d. Twenty-two minutes: Nuclear and cytoplasmic changes far advanced. Large area of monolayer denuded.

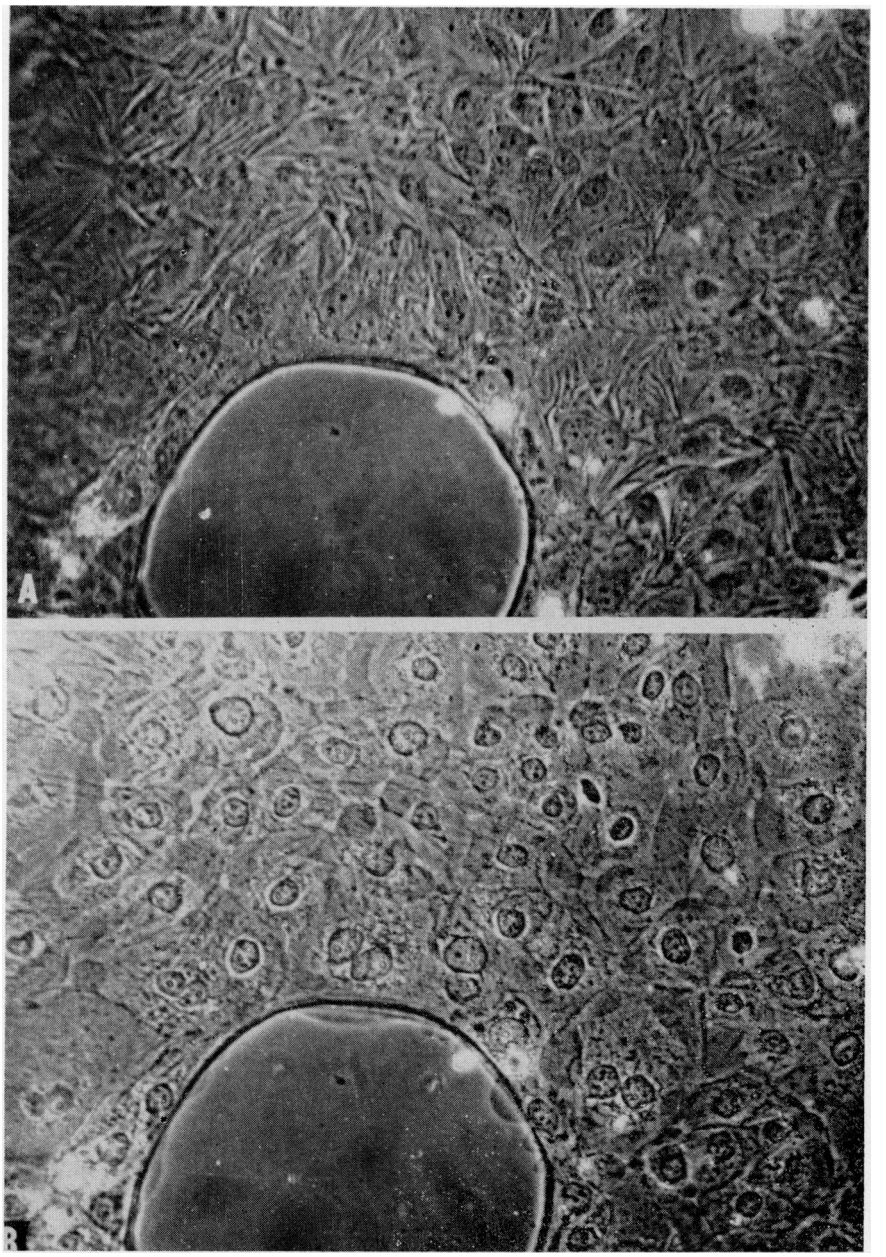

FIG. 2. Human amnion tissue culture after addition of purified staphylococcal α-toxin. Glass cover-slip preparation. Single field. Phase-contrast microscopy. 200x magnification.

a. Five minutes: No changes. At bottom of field is area without cells, an artefact in the monolayer.

b. Twenty-two minutes: Nuclear changes, with loss of normal homogeneous appearance, thickening of nuclear limiting membrane and formation of clear zone. Cytoplasmic extrusions are visible extending into cell-free area.

The effect of α-toxin on the release of virus from human amnion and rabbit kidney cells was determined in the following manner: The tissues were infected with the virus and 24 hours later, when CPE was evident in about 25 per cent of the cell sheet, were washed twice with Hanks' balanced salt solution. One milliliter of purified toxin (640 hemolytic units per milliliter) was then added to each tube. Fluids from three or four tubes were pooled at specific intervals, centrifuged, and the supernate titrated for virus content

TABLE 2. RELEASE OF HERPES SIMPLEX VIRUS FROM RABBIT KIDNEY AND HUMAN AMNION CELLS EXPOSED TO PURIFIED STAPHYLOCOCCAL α-TOXIN

Tissue culture	*Time of exposure (minutes)*	*Dilution of virus causing CPE*	
		Toxin	*Control*
Rabbit kidney	5	10^{-3}*	10^{-4}
	15	$10^{-4.5}$	$10^{-3.5}$
	30	$>10^{-5.5}$	10^{-4}
	60	$>10^{-5.5}$	10^{-4}
	freeze-thaw	....	$10^{-4.5}$
Human amnion	½	10^{-3}	10^{-3}
	1	10^{-3}	$10^{-2.5}$
	2	10^{-3}	$10^{-3.5}$
	5	$10^{-3.5}$	10^{-3}
	30	$10^{-3.5}$	$10^{-3.5}$
	60	$10^{-3.5}$	10^{-3}
	120	$10^{-3.5}$	10^{-3}
	freeze-thaw	....	10^{-4}

* Virus titer per 0.1 ml. of supernate.

in human amnion by the method described above. One control consisted of viral-infected cells which had been exposed to heated α-toxin; another was a group of cultures not exposed to toxin but frozen and thawed several times at the end of the incubation period. Additional cell damage produced by α-toxin was evident in both human amnion and rabbit kidney tissue in which CPE due to the activity of *herpes simplex* virus was present at the time the toxin was added. Disruption of the amnion monolayer did not occur.

Despite the development of cell damage, there was no significant difference in the yield of *herpes simplex* virus in human amnion cells treated with heated and unheated toxin. Rabbit kidney cells infected with herpes and exposed to toxin released a greater concentration of virus than control

cultures. The increase in concentration of virus roughly paralleled the degree of tissue damage produced by toxin (Table 2).

The effect of purified α-toxin on hemagglutination by, and infectivity of, influenza virus (PR 8 strain) was studied using human type 0 erythrocytes and embryonated hen's eggs. As can be seen in Table 3, the toxin was found to be without effect on the activity of the virus.

RELEASE OF S^{35}-L-METHIONINE INCORPORATED INTO CELLULAR PROTEIN

Sufficient quantitites of S^{35}-L-methionine were found to be incorporated into rabbit kidney and human amnion cell protein within 24 hours to permit measurement of the effect of α-toxin on release of the radioactive amino acid. Each experiment was carried out using 10 tissue cultures for each

TABLE 3. EFFECT OF STAPHYLOCOCCAL α-TOXIN ON HEMAGGLUTINATION AND EGG INFECTIVITY OF INFLUENZA PR-8 VIRUS

	Toxin	*Heated toxin*	*No toxin*
Hemagglutination	1:256	1:256	1:256
Egg infectivity (I.D. 50)	$10^{-5.5}$	$10^{-5.5}$	$10^{-5.7}$

time interval; the fluids from each group of cultures were pooled and assayed for radioactivity.

Effect of toxin on rabbit kidney cells. The results of a typical experiment are presented in Figure 3. After 5 minutes of exposure to toxin (hemolytic titer for rabbit erythrocytes-1 :600), there was an approximately equal degree of release of S^{35}-L-methionine from treated and control cells. As the duration of contact was prolonged, the quantity of radioactive amino acid escaping from the toxin-exposed cells increased and reached a maximum after 30 minutes; tissue damage at this time was maximal.

The results of studies of the release of TCA-precipitable S^{35}-L-methionine induced by exposure of cells to α-toxin are presented in Figure 4. The quantity of amino acid released as protein reached a peak (68 per cent) after 30 minutes. The total S^{35}-L-methionine content of untreated tissue cultures was about double that released by the addition of toxin. Disruption of control cell cultures by repeated freezing and thawing yielded 87 per cent of the available radioactivity; 78 per cent was present in the TCA-precipitable fraction.

Alpha-toxin of relatively low potency produced maximal effects after 60 rather than 30 minutes. The percentage of S^{35}-L-methionine released as

protein was, however, equal to that detected when more potent preparations were used.

Effect of toxin on human amnion cells. Studies of the effect of α-toxin on the release of S^{35}-L-methionine from human amnion cells yielded inconclusive results. The degree of visible damage was regularly less than that observed in rabbit kidney tissue. The results of a typical experiment in which

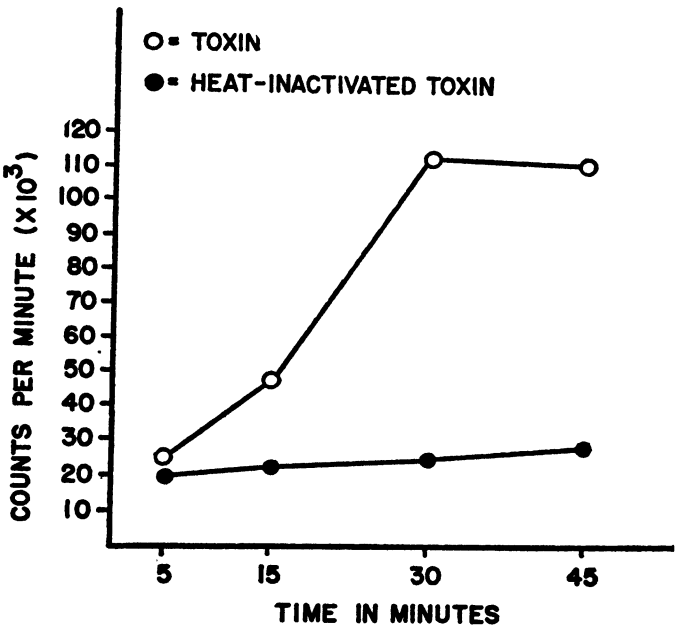

FIG. 3. Release of S^{35}-L-methionine from rabbit kidney cells exposed to purified staphylococcal α-toxin.

amnion cells were exposed to purified α-toxin (600 hemolytic units per milliliter) are illustrated in Figure 5. Although there was a slightly greater release of radioactive amino acid from the toxin-treated than from control cultures, the difference was always small and did not approach that observed when rabbit kidney cells were studied under the same conditions.

DISCUSSION

A number of investigators[5-9] have reported that the α-toxin of *Staph. pyogenes* (var. *aureus*) damages various cells growing in tissue culture. The effects of this agent on chick embryo cells have been described by Nogrady and Burton;[5] sudden contraction of the cytoplasm and nucleus was observed after a latent period of up to 20 minutes. Extrusion of cell

contents prior to contraction was not noted. Gabliks and Solotorovsky[6] found that staphylococcal α-toxin produced cytopathic effects in 24 hours in a variety of rabbit, human, and mouse cell cultures; they did not record the nature of the changes, however. Necrosis of human and guinea pig skin explants was found to result from contact with toxin for 3 days.[7] Injury of human esophageal epithelium and rabbit fibroblasts by exposure to α-toxin for 4 hours has been reported by Thal and Egner.[8] The human cells, although dead, did not separate from the glass surface on which they were multiplying; the fibroblasts, on the other hand, underwent a "4+ cell loss."

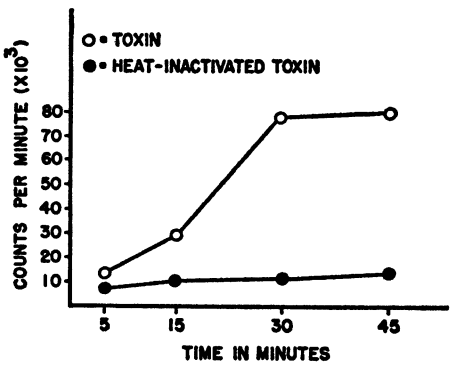

FIG. 4. Release of TCA-precipitable S^{35}-L-methionine from rabbit kidney cells exposed to purified staphylococcal α-toxin.

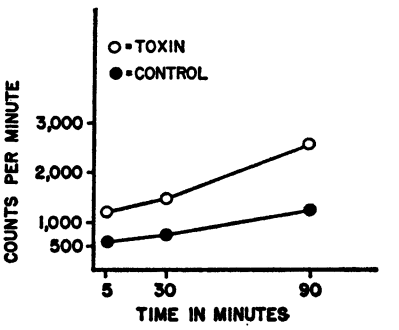

FIG. 5. Release of S^{35}-L-methionine from human amnion cells exposed to purified staphylococcal α-toxin.

The preparations of staphylococcal α-toxin studied by the investigators cited above were relatively crude. The results of the studies described in this paper suggest that the cytotoxic effects observed with the impure materials were, in all probability, due to activity of α-toxin since the highly purified substances produced similar changes. In the present investigation, evidence of tissue damage appeared rapidly in rabbit kidney and was morphologically complete within 30 minutes after exposure to toxin. The effects on human amnion developed more slowly and were less intense.

The rapidity with which cellular changes were produced by staphylococcal α-toxin in cultures of rabbit kidney suggested the operation of a mechanism similar to that responsible for hemolysis of rabbit erythrocytes. The release of intracellular protein and virus from kidney cells and the difference in reactivity of human amnion and rabbit kidney also suggested a similarity to the action on red blood cells. The difference in hemolytic activity of α-toxin on human and rabbit erythrocytes is well established.[4] Whether the observed species differences in sensitivity to α-toxin is a qualitative

or quantitative phenomenon is not known. Composition of the cell wall may be the critical factor. This has been found to differ in red blood cells of various species.[14] This difference may determine the ease with which α-toxin produces rupture of the cell wall and release of intracellular substances.

SUMMARY

Rabbit kidney cell cultures developed cytoplasmic extrusions and nuclear changes after exposure to highly purified staphylococcal α-toxin. The effects were rapid and were associated with increased release of *herpes simplex* virus and intracellular protein labelled with S^{35}-L-methionine, suggesting direct damage to cell membrane by α-toxin. Human amnion cell culture also showed morphologic changes and were killed by the toxin. Significant increases in the release of intracellular substances from amnion cells were not demonstrated. The difference in the effects of α-toxin on these tissues may be related to differences in the composition of their cell membranes.

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