THE SITE OF ACTION OF THE STAPHYLOCOCCUS ALPHA TOXIN*

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PLATE 20

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Staphylococcus pyogenes infection in both man and the lower animal is characterized by local tissue death and abscess formation. Of the many substances elaborated by this microorganism, the alpha toxin appears to be that most closely connected with tissue destruction (1), although the precise role of this substance in the pathogenesis of abscess formation is not yet clear (2). Systemic vascular effects of staphylococcus infection have also been ascribed to the action of the alpha toxin (3). Knowledge of the mechanism of action of this toxin would appear to hold fundamental implications for the understanding of staphylococcus infection in man. The studies now to be reported demonstrate that the alpha toxin acts selectively on the smooth muscle cell producing an initial prolonged contraction and subsequent paralysis. It will be shown that the local and systemic effects of the toxin can be explained in whole or in part by this action.

Materials and Methods

Previous studies (1, 4) have suggested but not established the selective effect of staphylococcus toxin on smooth muscle cells. The present experiments were designed to study this effect on isolated segments of smooth muscled structure *in vitro* and *in vivo*. In brief, the following studies were made.

1. The effect of staphylococcus toxin on the intraluminal pressure of isolated segments of small bowel and various blood vessels.
2. The response of isolated smooth and striated segments to stimulation before and after exposure to staphylococcus toxin.
3. The structural changes in blood vessels after exposure to staphylococcus toxin.
4. The changes in vascular resistance in isolated, perfused organs after administration of toxin.
5. The effect of staphylococcus toxin on various cells grown in tissue culture.

Animals.—Adult 12 to 20 kg. mongrel dogs and market bred 2 to 3 kg. albino rabbits were used.

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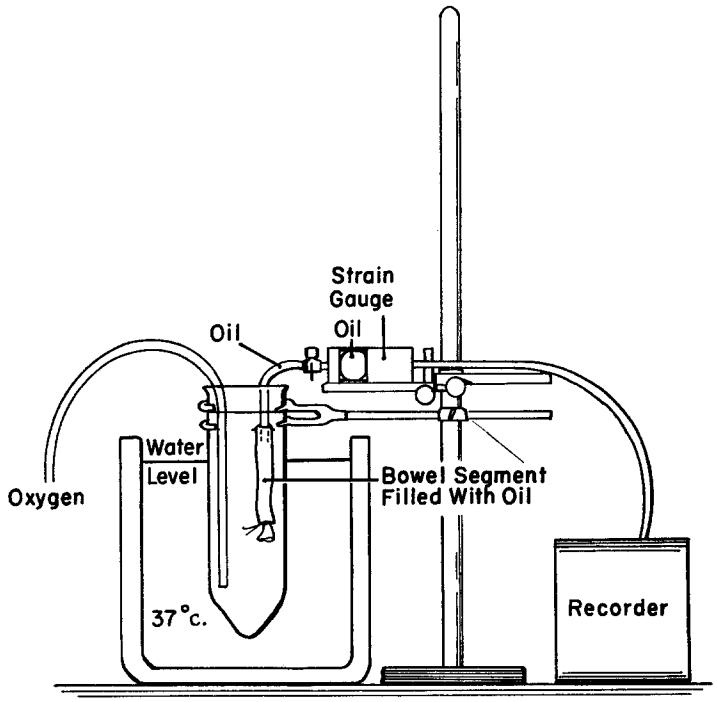
Preparation and Standardization of Toxin.—The staphylococcus toxin used was derived from 5-hour cultures of the Wood 46 strain and other pathogenic human strains. The methods for preparation and standardization have been previously described. (1).

Antitoxin.—Specific horse antitoxin containing 800 units per ml. was used.[1]

Blood Vessel Preparation.—The preparation most commonly used was the inferior vena cava of the rabbit. Using standard surgical techniques, a segment about 2 cm. in length was removed from the living animal under general anesthesia. This generally included the renal vein



TEXT-FIG. 1. Apparatus used for recording changes in spontaneous peristaltic activity in isolated duodenal segments.

branches. All branches were carefully ligated; a glass-tipped catheter was tied into one end, and the segment was flushed with saline and ligated at its distal end. The vessel segment was then filled with mineral oil and the catheter then connected to a Statham low pressure strain gauge by means of polyethylene tubing. The vein segment was then suspended in Ringer's solution at 37° C., through which oxygen was continuously bubbled. Continuous recordings of the intraluminal pressure were made using the Sanborn twin-viso. Other studies were recorded in the same way using the carotid arteries and vena cava of the dog.

The Intestinal Preparation.—2 cm. segments of duodenum and jejunum were taken from the living rabbit, carefully flushed with saline, cannulated at one end, and filled with sterile mineral oil. The distal end was then ligated and the cannula connected to the strain gauge system as described above. (Text-fig. 1).

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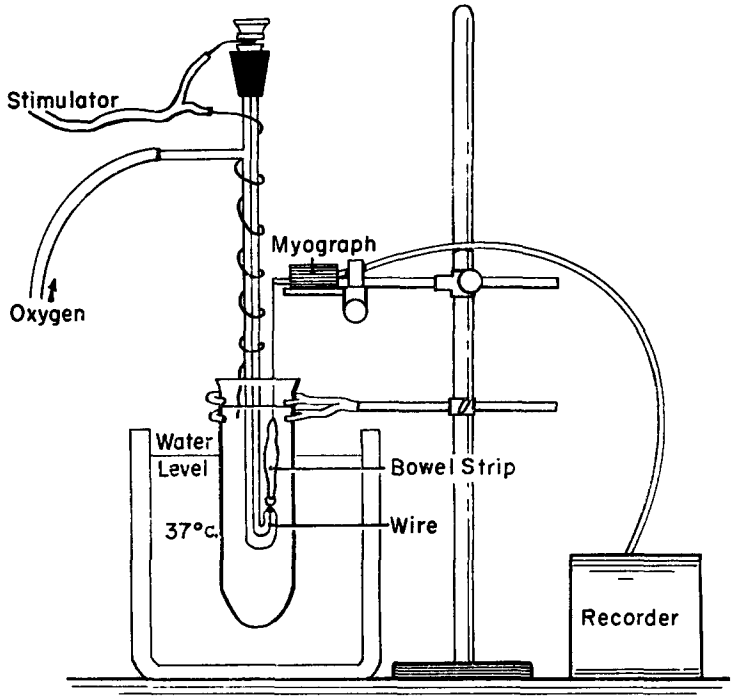
[1] Supplied by Lederle Laboratories, Pearl River, New York.

Muscle Strip Preparation.—1 cm. × 2 mm. segments of the thigh muscle of the rabbit were removed and a short piece of heavy silk suture was attached to one end and looped over the vertical armature of a Statham myograph. The other end was attached by metal skin clip to an electrode leading to a Grasse stimulator. The muscle was then suspended in Ringer's solution at 37° C., and the myograph was connected to the recording equipment. Small cardiac segments and uterine segments of the rabbit were studied in the same manner (Text-fig. 2).



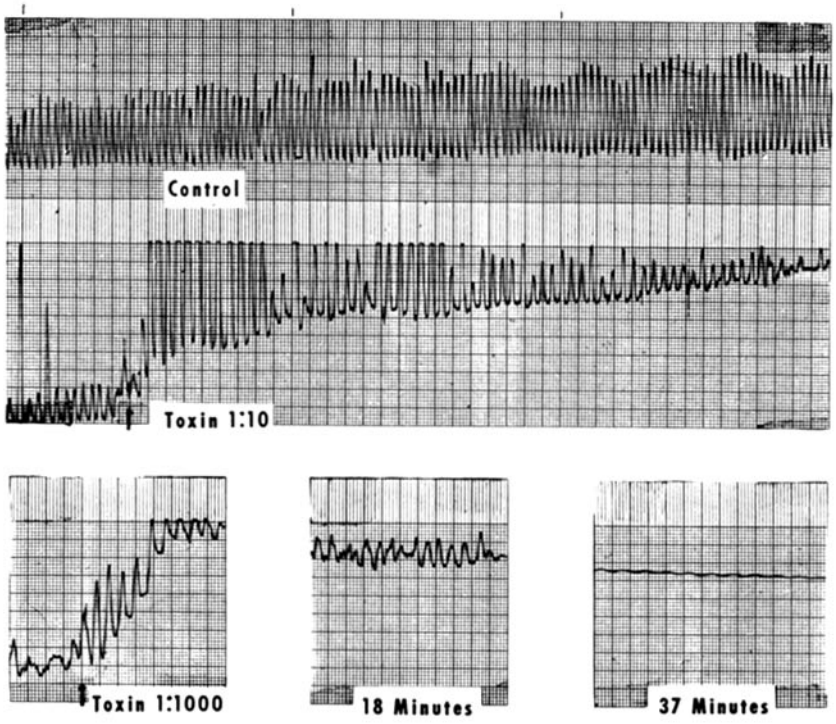
TEXT-FIG. 2. Apparatus used for recording tension changes in strips of bowel wall, cardiac muscle, and skeletal muscle.

Perfusion of Isolated Dog Kidney and Heart.—A large donor dog was heparinized and the carotid and jugular veins cannulated. A smaller animal was anesthetized and the aorta cannulated, either through the subclavian, for cardiac perfusion, or at the level of the renal arteries, for kidney perfusion. The relevant branches of the aorta were then ligated and divided and the donor dog allowed to perfuse the heart or kidney *in situ*. As soon as this perfusion was started, the organ was rapidly removed and suspended over a funnel leading to a reservoir from which the venous blood was pumped back to the donor. Blood flow was measured by timed collection of venous drainage from the perfused organ. The perfusion pressure was constantly measured by a pressure transducer connected to the arterial line. Resistance was calculated from the ratio of pressure gradient to flow and expressed as simple units of resistance. Toxin was injected into the arterial line immediately proximal to the perfused organ.

Tissue Culture Studies.—Human esophageal epithelium and rabbit fibroblasts were grown in human serum by standard cell culture techniques using established cell lines dispersed by trypsinization.

EXPERIMENTAL

1. Spasm and Subsequent Paralysis Produced in the Isolated Bowel Segment after Exposure to Staphylococcus Toxin.—Control studies demonstrated that bowel segments prepared in the manner described above would maintain regular peristaltic activity for periods exceeding 60 minutes. Various concentrations of staphylococcus alpha toxin were added to the bathing fluid. A

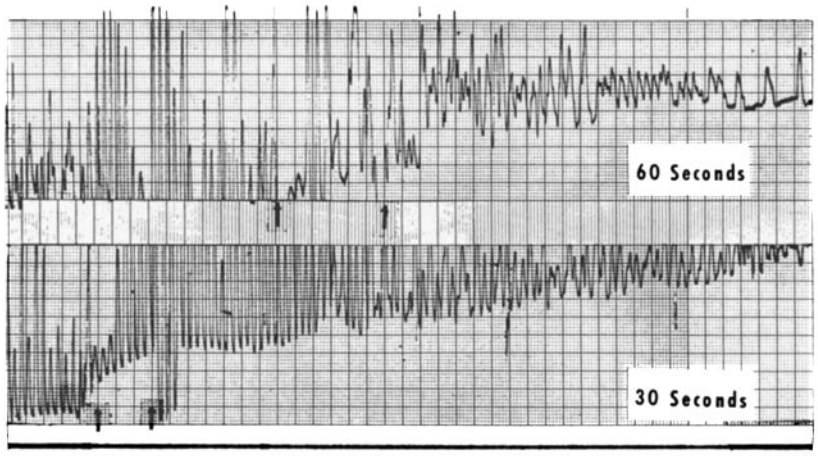


TEXT-FIG. 3. Comparison of peristaltic activity in control duodenal segment suspended in Ringer's solution and segments exposed to dilutions of staphylococcus toxin.

profound effect on intraluminal tension was regularly recorded within a few seconds after exposure to alpha toxin. After a few initial contractions of high amplitude, there was a rapid rise in resting intraluminal tension, with a progressive diminution in the rate and amplitude of contraction. Depending on the amount of toxin added to the bathing medium, there occurred a complete disappearance of peristaltic activity within a period varying from 4 to 12 minutes. The intraluminal tension, however, persisted at a high level for as long as 30 minutes before there was a progressive fall to levels below the base line (Text-fig. 3). Direct observation of the bowel segment revealed an intense

spasm at the height of this reaction. This terminated in progressive atony and finally flaccid distension. The reaction was not spontaneously reversible, for there was no recovery of peristaltic activity even after long periods of observation. The effect of varying concentrations of staphylococcus toxin on this experimental preparation was less pronounced but definite, and similar effects were found with a final dilution as high as 1:1000. It is apparent that high dilutions of this toxin have a profound effect on smooth muscle contractility.

2. Rapid and Irreversible Interaction between Staphylococcus Toxin and Smooth Muscle.—Using the same preparations, the bowel loops were exposed over periods of 30 to 60 seconds to the alpha toxin. The loops were then thor-

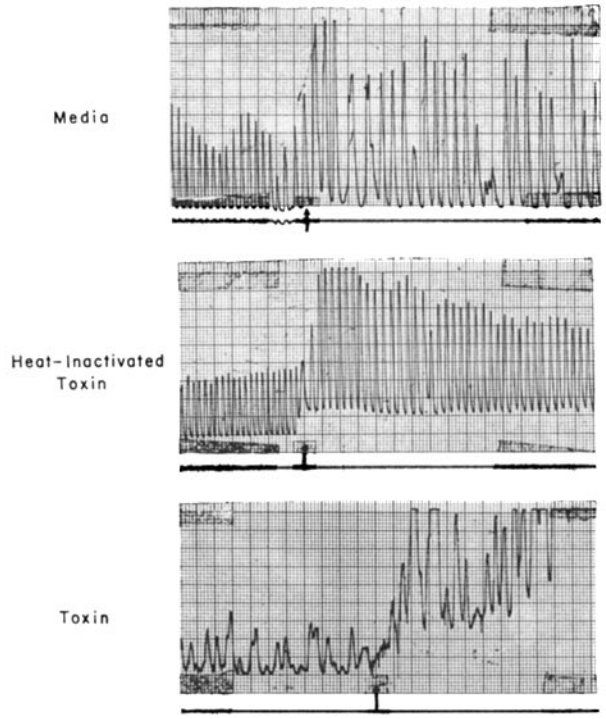


TEXT-FIG. 4. At the points indicated, staphylococcus toxin was added for 30 or 60 seconds to the bathing fluid. The fluid was then changed, the loops washed in fresh Ringer's solution, and replaced. The characteristic response is still observed.

oughly and rapidly washed in Ringer's solution. Recordings of such an experiment are shown in Text-fig. 4. It will be seen that exposure for as short a time as 30 seconds resulted in the same irreversible change, as is seen after prolonged exposure to toxin.

3. Evidence That the Toxic Effect on Smooth Muscle is Produced by Staphylococcus Alpha Toxin.—In working with an as yet chemically uncharacterized toxin, which is simply a culture filtrate, the possibility that biologic effects are produced by the media, endotoxic derivatives of cell walls, or histamine-like substances must be excluded. Accordingly, control studies were made by adding culture medium to the bathing fluid and by inactivating the alpha toxin by heating or by the addition of specific antitoxin (Text-fig. 5). In addition, a comparison was made of the effects of a known endotoxin (*Escherichia*

coli, Difco Laboratories) and histamine on isolated smooth muscle preparation (Text-fig. 6). The finding that the hemolytic, dermonecrotic, and myotoxic effects of the alpha toxin could be removed by heating for 60 minutes at 60°C. and completely neutralized by specific antitoxin, helped differentiate this substance from the relatively heat-stable histamine and endotoxin. Moreover, neither histamine nor endotoxin produced an effect on smooth muscle com-

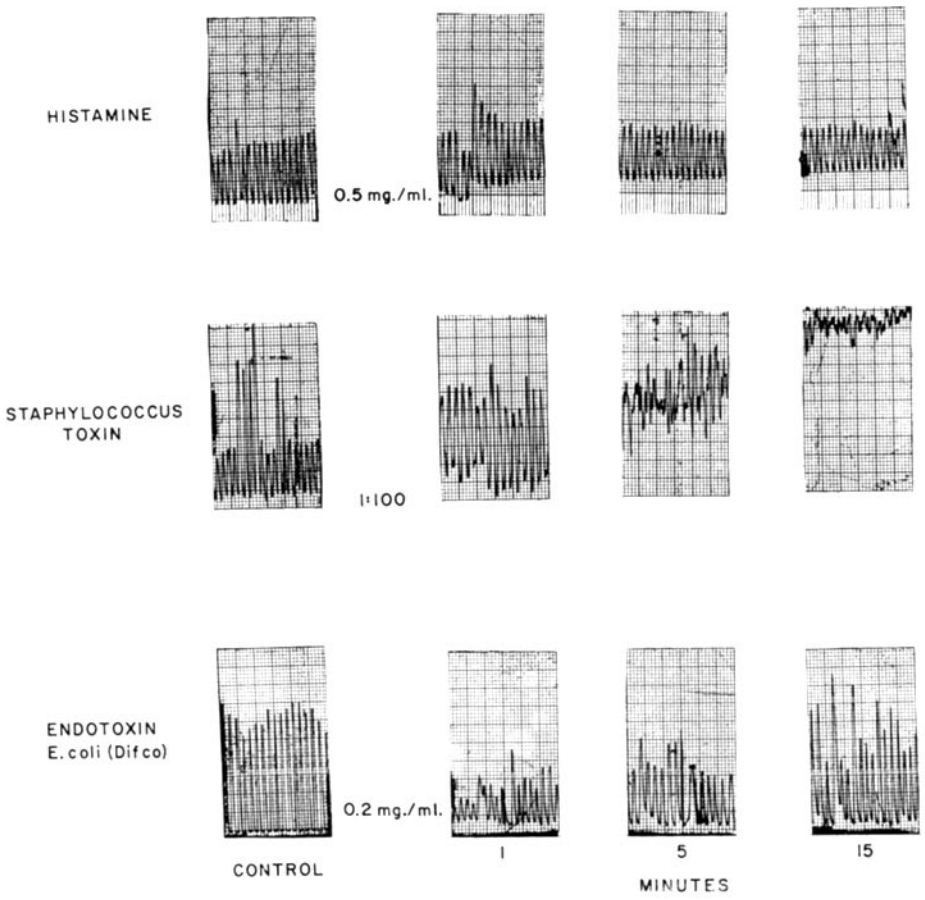


TEXT-FIG. 5. Comparison of peristaltic activity in duodenal segments exposed to culture medium, heat inactivated toxin, and active toxin.

parable to that of the toxin. It appears then that the myotoxic effects of staphylococcus toxin are brought about by a substance other than histamine or endotoxin.

4. The Protective Effects of Specific Antitoxin.—Using the same experimental arrangement as is described above, 800 units of specific antitoxin were first added to the bathing solution around the peristalting loop of duodenum. The subsequent addition of large amounts of toxin to this bathing fluid failed to produce the characteristic change described above, although some transient hyperperistalsis was seen (Text-fig. 7). This is probably accounted for by slight contamination of the toxin with histamine. On the other hand, when

antitoxin was added after exposing the bowel loop to toxin, no particular inhibitory effect on the action of the toxin could be found, even when the time interval between toxin and antitoxin exposure was as short as 30 seconds. These latter two experiments appear to indicate that the toxin is almost in-

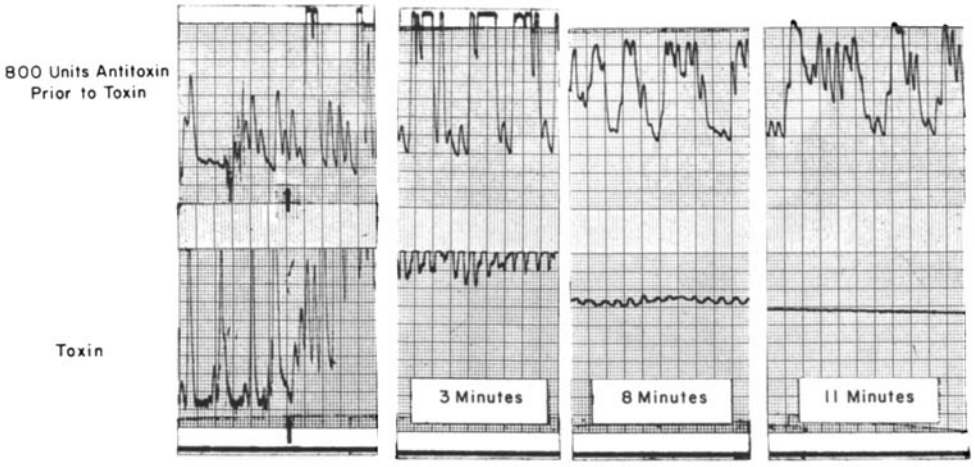


TEXT-FIG. 6. The effects of histamine and *E. coli* endotoxin are compared with staphylococcus toxin. The endotoxin produces a transient depression of peristaltic activity and the histamine, a slight stimulation.

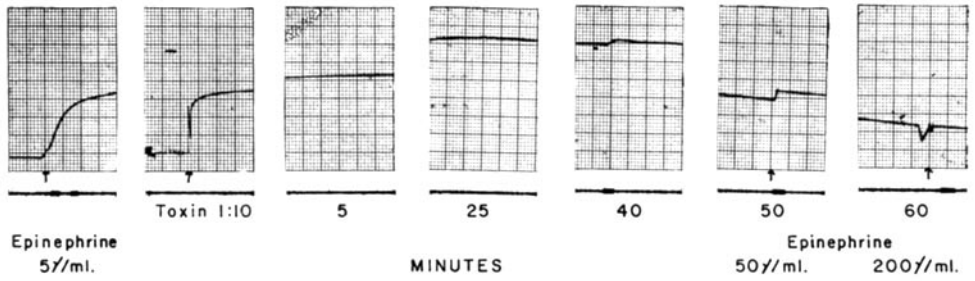
stantaneously and irreversibly bound to its substrate. This rapid and irreversible reaction has implications for understanding the failure of antitoxin clinically in the face of established infection. Of further interest is the finding that when the bowel segment is inverted so that the mucosal surface is bathed in toxin, no effect on peristaltic activity can be detected.

5. *The Effect of Toxin on Isolated Blood Vessel Segments.*—A similar experi-

ment was carried out using isolated 2 cm. segments of rabbit inferior vena cava. The intraluminal pressure was measured before and after addition of toxin to the bathing fluid. There was a progressive rise in intraluminal tension followed by atony, and a fall below base line levels, at which time the vessel segment was no longer responsive to epinephrine stimulation (Text-fig. 8).



TEXT-FIG. 7. The protective effect of specific antitoxin is demonstrated here. The arrow indicates point at which toxin was added.



TEXT-FIG. 8. The vena caval segment was filled with oil and intraluminal pressure measured. The initial response to 5 gamma epinephrine is shown. Toxin was then added to the bathing fluid. The initial increase in intraluminal pressure and subsequent fall, is shown. Also note the failure to respond to epinephrine terminally.

The ultimate paralysis and flaccid distension of these caval segments may hold implication for the understanding of the lethal action of the staphylococcus toxin. The clinical picture as shown previously (3) is one of inadequate venous return to the heart. This is due only in part to splanchnic and hepatic

sequestration of blood. It now appears more likely that generalized venous pooling brought about by direct vasoparalytic effect of the toxin is the basis of the lethal effect. It is also of some interest that the initial effects of a lethal dose of staphylococcus toxin is also seen on smooth muscle structures other than blood vessels. There is spasm of the iris, extreme hyperperistalsis of the gut. This is followed in about 20 minutes by wide dilatation of the iris and atony and progressive distension of the bowel.

6. Structural Changes in the Walls of Muscular Blood Vessels Exposed to Staphylococcus Toxin.—Previous studies in this laboratory have shown that sublethal doses of staphylococcus toxin given intravenously produce renal cortical necrosis in rabbits. In its initial stages, this lesion appears to be the result of selective renal vasospasm. In animals surviving over 24 hours, histologic examination shows destruction of the muscular media of the arcuate arteries. Taken together with the experiments described above, this would seem to demonstrate a selective effect on smooth muscle cells. To study this phenomenon further, staphylococcus toxin was infiltrated in the neighborhood of several mesenteric arteries and the femoral artery and vein in the rabbit. In all animals killed after 24 hours, the muscular media was found to be transformed into a homogeneous anuclear zone, although the intimal cells and periadventitial connective tissue cells appeared to be viable. This structural change is illustrated in Fig. 1. In some instances, thrombosis of such vessels ultimately developed, although it appeared quite clear on histologic grounds that this was a late development.

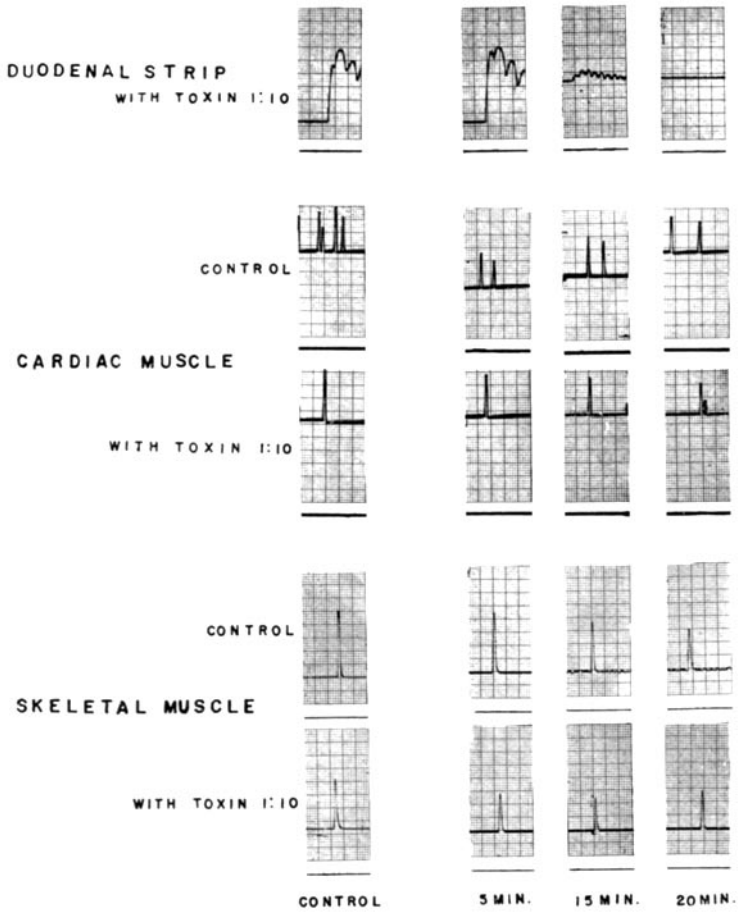
7. A Comparison between the Effects of Staphylococcus Toxin on Smooth Muscle, Skeletal Muscle, and Cardiac Muscle.—Since no clear cut biochemical differences have yet been described between the contractile protein of smooth muscle and striated muscle, a study of the action of the toxin on these different types of muscle seemed indicated.

To perform this experiment, a technique slightly modified from that described above was used. This method is illustrated Text-fig. 2. Strips of rabbit sartorius muscle and cardiac and duodenal muscle were aligned between the myograph terminals through which an electric shock of 30 volts could be passed. The muscles were suspended in continuously oxygenated Ringer's solution at 37°C. Control studies of such preparations revealed that reproducible responses to shock could be obtained over a 45 minute period. The results of such an experiment are shown in Text-fig. 9.

It will be seen that the addition of staphylococcus toxin to the bathing fluid had no detectable effect on the electrically-induced contraction of either the skeletal muscle or the cardiac muscle. However, in the smooth muscle preparation, a reaction entirely similar to that described previously is seen. A preliminary study carried out by us failed to show any distinct alterations in the viscosity of contractile protein extracted from smooth muscle or skeletal

muscle after exposure to toxin. This may indicate that the site of action of the toxin is on the smooth muscle nucleus or the cytoplasmic membrane.

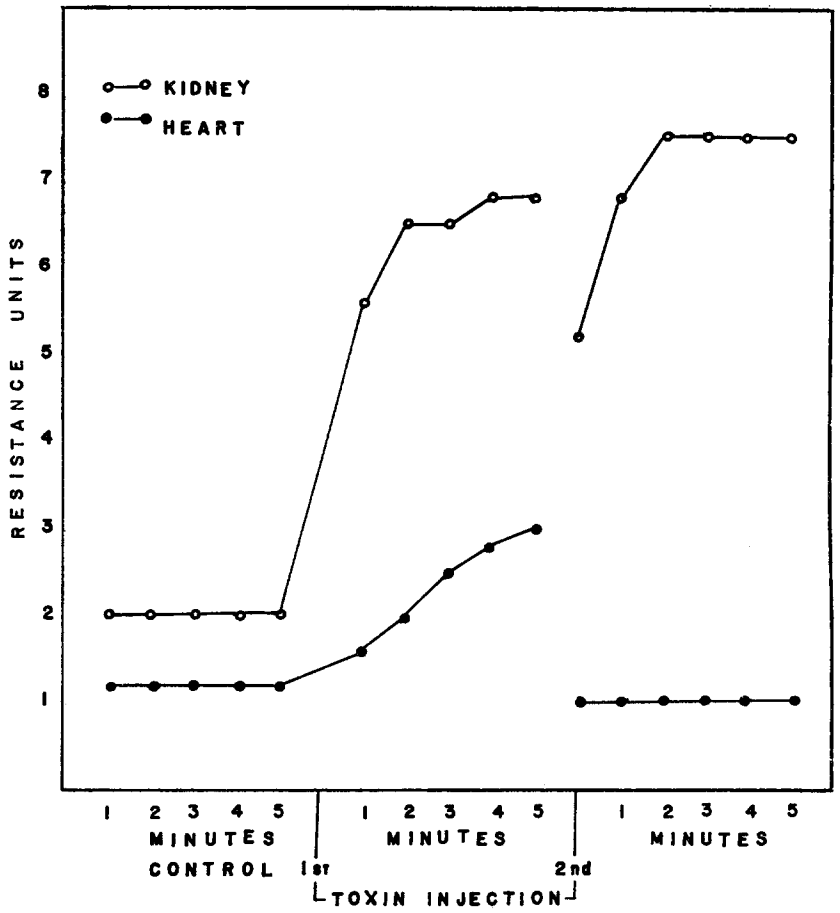
8. Increased Resistance to Perfusion of Isolated Kidney and Heart Preparations after Exposure to Staphylococcus Alpha Toxin.—Previous studies have shown



TEXT-FIG. 9. Strips of duodenal, skeletal, and cardiac muscle were arranged in the apparatus sketched in Text-fig. 2. Control responses to a standard electric shock before and after exposure to staphylococcus toxin are shown. Only the smooth muscle showed an increase in resting tension and failed to respond to subsequent electric shock.

that the blood flow to organs exposed to toxin is diminished. When the substance is given intravenously to rabbits, in suitable amounts, there appears to be a selective vasospastic effect on the kidneys, which results in various degrees of renal cortical necrosis (4).

To quantitate these changes, the isolated dog kidney and heart preparations were studied. The results of these experiments are shown in Text-fig. 10. It will be seen that the resistance increases almost immediately after injection of toxin. To exclude the possibility that the change in resistance was brought



TEXT-FIG. 10. The freshly removed kidney or heart was perfused with oxygenated blood. The addition of toxin to the perfusate regularly produced a striking fall in flow. This is documented by expressing resistance as the ratio of flow to pressure gradient across the perfused organ.

about by red cell agglutination, the experiment was repeated using dog plasma for perfusion. Identical changes in resistance were found. The possibility of interstitial edema contributing to the changes in resistance was eliminated by a continuous recording of the weight of the kidney during perfusion; there

was no significant early change in weight. These findings emphasize the profound vasospastic effects of the alpha toxin, and taken together with the results of the experiments on isolated bowel and blood vessels indicate that this substance has a direct effect on blood vessels which is mediated neither by nervous nor humoral mechanisms. Moreover, these perfusion studies emphasize the special sensitivity of the renal vessels to this substance.

TABLE I

Effect of Staphylococcus Toxin on Tissue Culture*

Cells *in vitro*	Exposure time	Morphologic effect	Lethal effect‡
Control§	4 hrs.	None	None
Human esophageal epithelial cells	30 min.	None	None
	60 min.	"	"
	2 hrs.	Slight granularity	"
	4 hrs.	1+ rounding of cells	
		1+ granularity	
		No loss in cell number	4+
Control§	4 hrs.	None	None
Rabbit fibroblasts	30 min.	None	None
	60 min.		
	2 hrs.		
	4 hrs.	1+ vacuolization	
		4+ granularity	4+
		4+ cell loss	

* 1:10 dilution was used.

‡ As evidenced by failure of cell recovery and metabolism.

§ Control—Cell culture medium plus culture medium used for growth of staphylococcus.

9. The Effect of Staphylococcus Alpha Toxin on Human and Rabbit Cells Growing in Tissue Culture.—Some workers have suggested that the alpha toxin is a general cellular poison (5–7). Our studies, on the otherhand, suggest a preferential site of action on the smooth muscle cell. To test this hypothesis further, cell cultures of human esophagus epithelium and rabbit fibroblast cells were exposed to staphylococcus toxin in various concentrations, then resuspended in tissue culture medium.

The results of this study are shown in Table I. It will be seen that the effect on these cells in tissue culture is minimal. After the cells are bathed in toxin for 2 hours they recover and metabolize when transferred to growth medium. On the other hand, exposure for 4 hours was lethal to the cells. These findings again emphasize the special sensitivity of smooth muscle cells which are ir-

reversibly damaged after an extremely short exposure to the action of the alpha toxin.

DISCUSSION

The host reaction to staphylococcus infection may take one or more of several different forms. These are, in general, characterized by pus formation and tissue necrosis and the inflammatory reaction may vary from a mild erythema to a massive gangrenous necrosis. Certainly, the various constituents of the bacterial cell as well as diffusible products produce the final host reaction. Nevertheless, the weight of evidence favors the concept that the alpha toxin, so potent in its destructive abilities, plays an important role in the necrotizing form of staphylococcal disease.

Previous studies by others (6, 7) have suggested that this substance is a general cytoplasmic poison, yet as indicated in the present study, epithelial cells in tissue culture and isolated preparations of living cardiac and skeletal muscle are resistant to its action. On the other hand, smooth muscle regardless of its origin, appears to be exquisitely sensitive, and is irreversibly damaged in a few seconds.

When the alpha toxin is injected intravenously in sublethal amounts, the most pronounced vascular effect is seen in the kidney (4). The present study confirms this finding by demonstrating the dramatic increase in renal vascular resistance after small amounts of toxin are given to the isolated perfused kidney. While the experimental situation is highly artificial, nevertheless, severe renal insufficiency is not uncommonly seen in overwhelming staphylococcus infection.

The studies of Ekstedt (8), Rogers and Tompsett (9), and Tager (10) have emphasized the role of coagulase and leukocidic factors in establishing the growth of the staphylococcus in the host. More recently, it has become evident that even coagulase-negative organisms may cause fatal staphylococcal endocarditis (11). The role of the alpha toxin in producing tissue destruction is thus only one facet of the complex interaction between the host, the microorganism, and its diffusible products.

SUMMARY

The present study concerns the site of action of the staphylococcus alpha toxin. This powerful necrotizing agent produced by pathogenic strains of staphylococcus is very probably important in the pathogenesis of localized staphylococcus disease and in the shock-like picture sometimes associated with staphylococcus septicemia. Our previous studies had suggested that the toxin has a selective effect on vascular smooth muscle.

In investigating this problem further, the following observations were made.

1. The toxin produces an immediate hyperperistalsis and sustained increase in intraluminal tension progressing ultimately to atony and flaccid paralysis in the isolated smooth muscle preparation.

2. The addition of specific antitoxin prior to exposure to toxin prevents this reaction. However, when antitoxin is added after the toxin, no ameliorating effect is seen.
3. The toxin is rapidly and irreversibly bound to its substrate since washing the bowel segment 30 seconds after exposure to toxin fails to change the course of the reaction.
4. Vena caval segments exposed to toxin exhibit a similar initial rise in intraluminal tension followed by flaccid paralysis at which point they no longer respond to epinephrine stimulation.
5. When the toxin is infiltrated in the neighborhood of muscular blood vessels in the living rabbit selective necrosis of smooth muscle cells of the vessel walls is seen.
6. The selective effect on smooth muscle is emphasized by the failure of the toxin to affect the contractility of skeletal and cardiac muscle.
7. Perfusion of the isolated kidney and heart produces an increased resistance to flow after toxin is added to the perfusate.
8. Epithelial cells and fibroblasts in tissue culture exposed to high concentration of toxin for 2 hours are unaffected in their ability to recover and metabolise. This is in marked contrast to the effect on the smooth muscle preparation.

The probability that the toxic effect on smooth muscle cells explains some of the local and systemic effects of staphylococcus infection is discussed.

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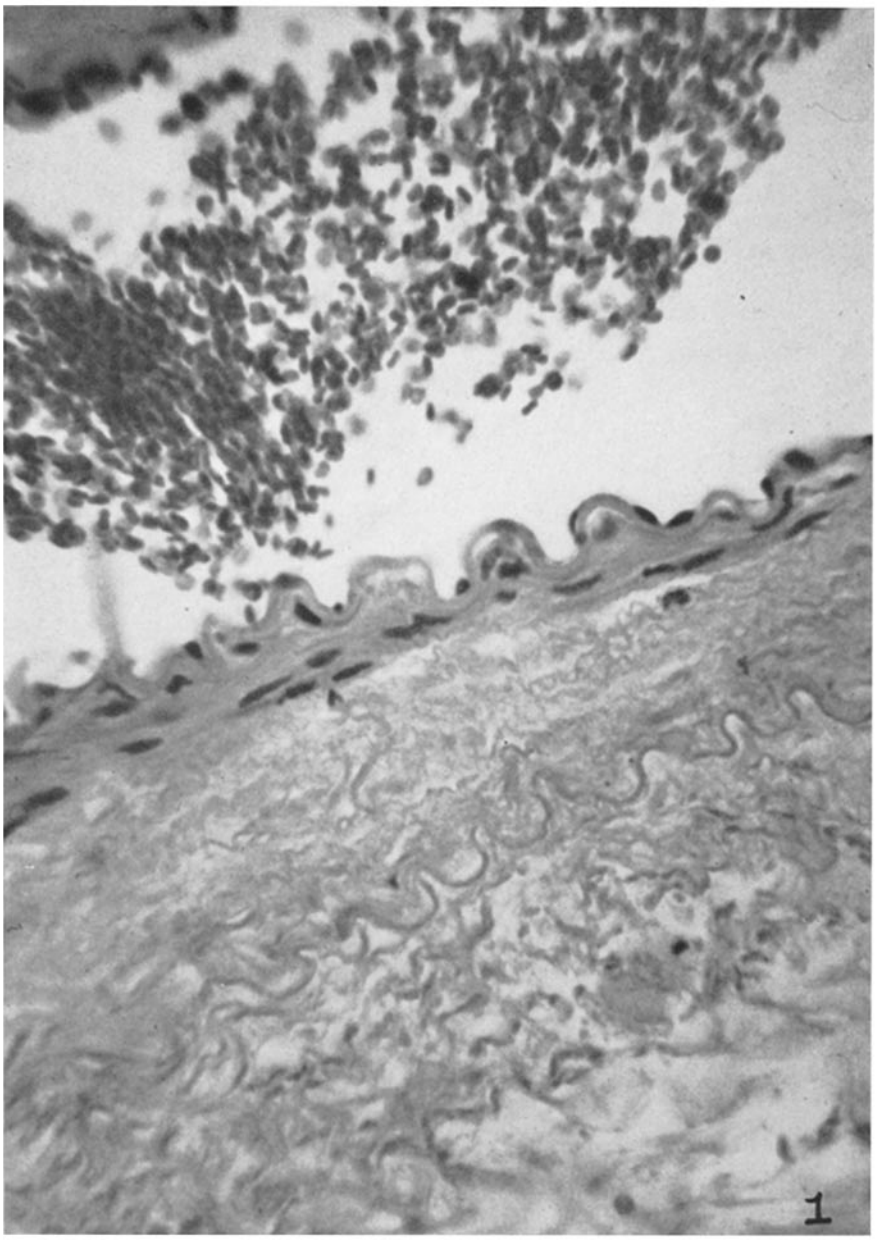
EXPLANATION OF PLATE 20

FIG. 1. Section of the rabbit femoral artery. Toxin was infiltrated in the vicinity of this vessel 24 hours before the animal was killed. Necrosis of smooth muscle cells is seen.



(Thal and Egner: Action site of staphylococcus alpha toxin)