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THE RELEASE OF HISTAMINE BY THE α-TOXIN OF *STAPHYLOCOCCUS PYOGENES*

BY

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Filtrates possessing a high α-haemolytic titre, obtained from cultures of *Staphylococcus pyogenes*, were reported by Feldberg & Keogh (1937) to release histamine from cat and guinea-pig perfused isolated lungs, and by Feldberg & Kellaway (1937) to release histamine and a slow-contracting substance from dog perfused lung and liver. Brown, Prichard & Quilliam (1959) were unable to detect the release by the α-toxin of any spasmogenic substance from segments of guinea-pig isolated ileum. Smith & Miles (1960) observed no increase in the amount of histamine in the peritoneal cavity of rats treated with staphylococcal α-toxin or with live staphylococci.

This paper reports the results of a further investigation into the possible histamine-liberating action of staphylococcal α-toxin, using the preparation of α-toxin employed previously (Brown *et al.*, 1959). The work forms part of an attempt to determine whether histamine release might play a role in the pharmacological actions of the α-toxin, such as the smooth muscle spasmogenic action reported by Brown *et al.* (1959) and by Wiegershausen (1962).

METHODS

Isolated Tissues

Tissues obtained from freshly-killed guinea-pigs and rats, or from anaesthetized cats, were cut into small pieces (about 0.5 cm^2, or, with skin, strips about 1 cm long by 1 to 2 mm wide), and incubated in 1 or 2 ml. of magnesium-free Tyrode solution at 37° C previously equilibrated with a mixture of 95% oxygen and 5% carbon dioxide. Between periods of incubation the tissues were immersed for a standard time, usually 90 sec, in a similar solution containing staphylococcal α-toxin, Compound 48/80, or no added agent (controls). After incubation the tissues were removed, blotted and weighed, and the incubation fluids were assayed for histamine content as described below.

Anaesthetized cats

Cats were anaesthetized with pentobarbitone (35 mg/kg, intraperitoneally) and carotid blood pressure was recorded with a mercury manometer. A femoral artery was cannulated and blood withdrawn into centrifuge tubes containing 100 units of heparin in 0.1 ml. before and after intravenous injection of staphylococcal α-toxin or Compound 48/80. The blood was centrifuged and the plasma was assayed for histamine content after suitable dilution with Tyrode solution.

Histamine assay

The isolated terminal ileum of the guinea-pig was superfused with magnesium-free Tyrode solution at 30° C, bubbled with a mixture of 95% oxygen and 5% carbon dioxide, and containing

0.01 μg/ml. of atropine. This fluid was dripped over the ileal segment, enclosed in a warming jacket, at a rate of 2.5 to 3 ml./min (about 60 drops/min). Standard histamine or test solutions (0.5 ml., warmed to 32° C) were dripped over the ileum from a tuberculin syringe for 15 sec at 90 sec intervals, during which the superfusion was arrested. Contractions of the ileum were recorded on moving smoked paper with a frontal writing lever.

Concentrations of histamine present in incubation fluids were estimated by comparing the responses of the ileum to different dilutions of each sample of fluid with those to standard concentrations of histamine, dose/response curves being constructed from the results. This method of assay was rapid and economical of solution. Abolition of ileal responses by mepyramine, and parallelism between the dose/response curves for dilutions of incubation fluid and for standard histamine concentrations served to identify the active substance in the incubation fluid as histamine-like. The total amount of histamine released during each incubation period was estimated and expressed as μg of histamine (as acid phosphate) per g of wet tissue. Amounts released by α-toxin or Compound 48/80 were calculated by deducting the amount released spontaneously from untreated tissues. Amounts of histamine released during the period of immersion in the solution of releasing agent were not estimated, because the presence of the agent interfered with the assay. Staphylococcal α-toxin tended to increase the spontaneous activity of the ileal segment, and occasionally produced a contraction. Compound 48/80, in concentrations greater than 1 μg/ml., exerted a marked and persistent depressant action. With the α-toxin the error in estimating total histamine release induced by this omission is unlikely to be great because of the delayed release (see Results and Fig. 3).

Drugs

Staphylococcal α-haemolysin (α-toxin) was from the same source (Wellcome Laboratories, Beckenham) as that used previously (Brown *et al.*, 1959). Doses are given in haemolytic units (H.U.), one H.U. being the least quantity required to lyse completely 1 ml. of a 1% suspension of fresh washed rabbit erythrocytes in 0.9% (w/v) saline. The antitoxin used was a pepsin-refined and concentrated (about threefold) serum preparation from immunized horses, free of antibodies to other staphylococcal haemolysins (Wellcome Laboratories). Doses of antitoxin are in international units (I.U.).

Doses of histamine acid phosphate, 5-hydroxytryptamine creatinine phosphate, atropine sulphate and mepyramine maleate refer to the weight of salt. Doses of Compound 48/80 (Wellcome Laboratories) refer to the pure substance.

RESULTS

Exposure of portions of guinea-pig isolated lung for 90 sec to staphylococcal α-toxin in concentrations of 0.02 to 2 H.U./ml. led to the subsequent release of histamine from the lung. The amounts of histamine released increased with increasing concentrations of α-toxin (Fig. 1).

Very little histamine was released from other guinea-pig isolated tissues by 90 sec treatment with 2 H.U./ml. of α-toxin, but substantial amounts of histamine were released from cat isolated skin (Table 1). No consistent liberation of histamine from rat isolated tissues was detected after application of 2 H.U./ml. of α-toxin for 90 sec, nor did direct application of α-toxin to isolated rat mesentery affect the ability of the mast cells to take up Toluidine Blue dye.

Compound 48/80 (1 mg/ml.) released more histamine than did 2 H.U./ml. of staphylococcal α-toxin from all of the tissues examined except from guinea-pig lung: from this tissue 2 H.U./ml. of α-toxin released six times more histamine than did 1 mg/ml. of Compound 48/80.

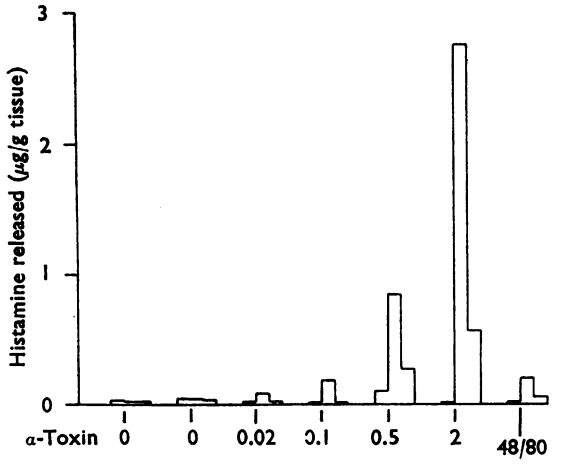

Fig. 1. Quantities of histamine (ordinate, μg/g of tissue) released from each of seven portions of isolated lung from the same guinea-pig during three successive 15 min incubation periods. The tissues were exposed for 90 sec between the first and second incubation periods (at the lines) to staphylococcal α-toxin (concentrations in haemolytic units/ml. indicated along the abscissa) or to 1 mg/ml. of Compound 48/80.

TABLE 1

AMOUNTS OF HISTAMINE RELEASED FROM ISOLATED MAMMALIAN TISSUES DURING A PERIOD OF 30 MIN AFTER EXPOSURE FOR 90 SEC TO STAPHYLOCOCCAL α-TOXIN (2 H.U./ML.) OR TO COMPOUND 48/80 (1 MG/ML.)

Each pair of values was derived from a single experiment. * Concentration of Compound 48/80, 10 μg/ml.

		Histamine released (μg/g of tissue)								
Tissue	Drug	1	2	3	4	5	6	7	8	Mean
Guinea-pig										
Lung	α-Toxin	0·73	1·94	0·79	4·59	1·27	3·30	1·94	4·68	2·41
	48/80	0·28	1·52	0·22	0·21	0·24	0·20	0·11	0·46	0·41
Skin (leg)	α-Toxin	0·92	0·51	0·10	0·13					0·42
	48/80	2·18	5·7	2·30	1·83					3·00
Skin (abdomen)	α-Toxin	0·08	0·20							0·14
	48/80	0·90	1·27							1·08
Skin (back)	α-Toxin	0·12								0·12
	48/80	0·24								0·24
Diaphragm	α-Toxin	<0·2	0·25							
	48/80	<0·2	0·55							<0·55
Ileum	α-Toxin	<0·1	<0·1	<0·1						
	48/80	<0·1	<0·1	0·25						<0·25
Uterus	α-Toxin	<0·03	<0·01							
	48/80	<0·03	0·03							<0·03
Cat										
Skin (leg)	α-Toxin	3·9	2·1	0·7	0·8					1·8
	48/80	28·6	38·9	7·1*	3·6*					33·7
Skin (abdomen)	α-Toxin	3·1	1·3	2·6						1·3
	48/80	12·4	7·3	7·7						9·1

Comparison of histamine release with spasmogenic action

The spasmogenic action of the α-toxin on isolated smooth muscle preparations shows the following characteristics (Brown *et al.*, 1959 ; Wiegershausen, 1962). (1) There is a delay between application of α-toxin and onset of the contraction ; with a concentration of 0.5 to 2 H.U. this delay lasts 90 sec or more in the guinea-pig isolated ileum. (2) The

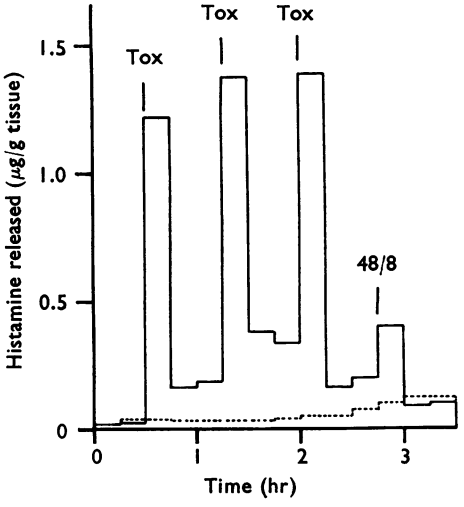

Fig. 2. Effect of repeated application of staphylococcal α-toxin on the guinea-pig isolated lung. The histogram shows the quantities of histamine released (ordinate, μg/g of tissue) from a portion of guinea-pig isolated lung during successive 15 min incubation periods. At the marks the lung tissue was exposed for 90 sec to 2 H.U./ml. of staphylococcal α-toxin (Tox) or to 1 mg/ml. of Compound 48/80. The quantity of histamine released spontaneously from a second, untreated portion of lung from the same guinea-pig is superimposed (broken line). Abscissa, time in hr.

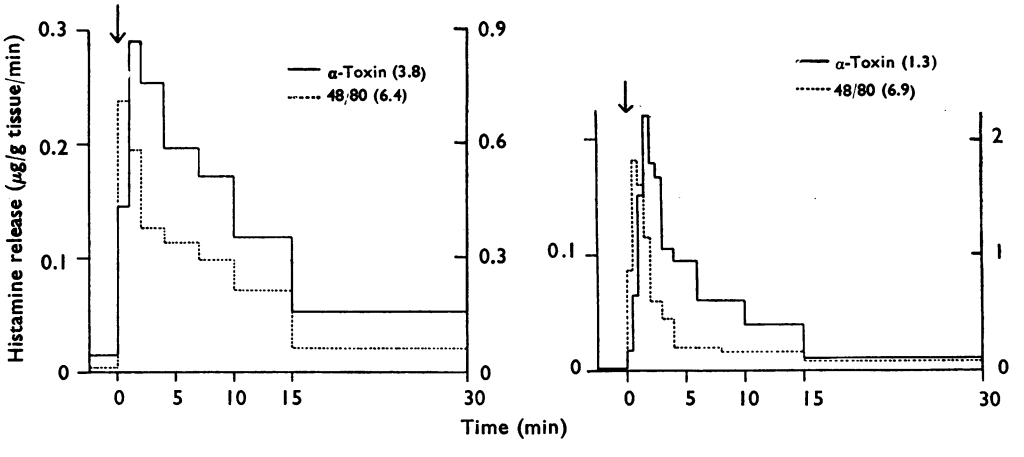

Fig. 3. Rate of histamine release (ordinates, μg/g tissue per min) from cat isolated skin following exposure for 20 sec (at arrows) to 2 H.U./ml. of staphylococcal α-toxin (full lines) or to 20 μg/ml. of Compound 48/80 (broken lines). Abscissae, time after withdrawal of skin from solution of releasing agent (min). The total amounts of histamine released from each portion of skin during 30 min incubation after exposure to the releasing agent are indicated by the figures in brackets (μg/g) on each graph. The two graphs depict results from two separate experiments, in each of which two adjacent strips of skin isolated from the inner aspect of the thigh of the same cat were used. Note that the ordinate scales pertaining to staphylococcal α-toxin (left-hand scales) and Compound 48/80 (right-hand scales) differ.

α-toxin must be left in contact with the tissue for a finite time, at least half the total delay time, for a contraction to occur. (3) A contractile response to the α-toxin cannot be elicited more than once in the same preparation. It was considered of interest to find out whether the histamine-releasing action of the α-toxin showed these characteristics.

Repeatability. When a piece of guinea-pig isolated lung was exposed repeatedly to 2 H.U./ml. of α-toxin for 90 sec periods at 45 min intervals, successively similar quantities of histamine were released (Fig. 2). Similar findings were obtained with cat isolated skin.

Time-course of release. After exposure of guinea-pig isolated lung or cat skin (Fig. 3) to staphylococcal α-toxin (2 H.U./ml.) for 20 sec there was a delay of about 90 sec before the ensuing maximal rate of histamine release. With Compound 48/80 the maximal rate of histamine release from cat isolated skin was attained within the first minute after removing the tissue from the Compound 48/80 solution. Relative to the total amounts of histamine released from the tissues during the 30 min period after application of the two agents, the maximal rate of release after application of Compound 48/80 was about twice that after staphylococcal α-toxin.

Contact time. The amounts of histamine released from portions of cat isolated skin and guinea-pig lung during a period of 30 min after exposure to 2 H.U./ml. were estimated. From cat skin the following quantities of histamine were released (one experiment): after 5 sec contact with α-toxin, 3.3 μg/g of tissue; after 15 sec contact, 3.2 μg/g; 45 sec, 1.5 μg/g; and 90 sec, 2.3 μg/g. From untreated skin 0.23 μg/g of histamine was released. The corresponding figures for histamine release from guinea-pig isolated lung (one experiment) were: untreated, 0.09 μg/g; 5 sec exposure, 3.9 μg/g; 15 sec, 3.0 μg/g; 45 sec, 6.6 μg/g; and 90 sec, 4.8 μg/g. These values do not include the histamine released during the periods of exposure to α-toxin, for the reasons given above (see Methods). However, from the results in Fig. 3 it appears unlikely that the quantities of histamine released during this period would have been more than 5 to 10% of the total released during the subsequent 30 min. Even after allowing for this error the results indicate that the amounts of histamine released by the α-toxin did not vary substantially with different periods of contact within the range of 5 to 90 sec.

Prevention of histamine release

Prolonged immersion of cat isolated skin in 1 mg/ml. of Compound 48/80 for 1 hr depleted the skin of between 80 and 90% of its histamine content (estimated by extracting the remaining histamine by heating to 100° C for 10 min), and prevented the subsequent liberation of further histamine by either Compound 48/80 or staphylococcal α-toxin. Warming the tissue to 55 to 57° C for 30 min also prevented the subsequent release of histamine by either agent: this procedure did not reduce the histamine content of the tissue by more than 4%. Warming to 45° C for 30 min did not reduce, but instead increased the amounts of histamine released after subsequent exposure of the skin to either Compound 48/80 or staphylococcal α-toxin. None of these procedures allowed any differentiation between the histamine-releasing actions of the two agents.

The histamine-releasing action of staphylococcal α-toxin was antagonized selectively by staphylococcal anti-α-toxin (Fig. 4). Treatment of either guinea-pig lung or cat skin with a mixture of 20 I.U. of antitoxin with 2 H.U. of α-toxin per ml. (Fig. 4, column 2)

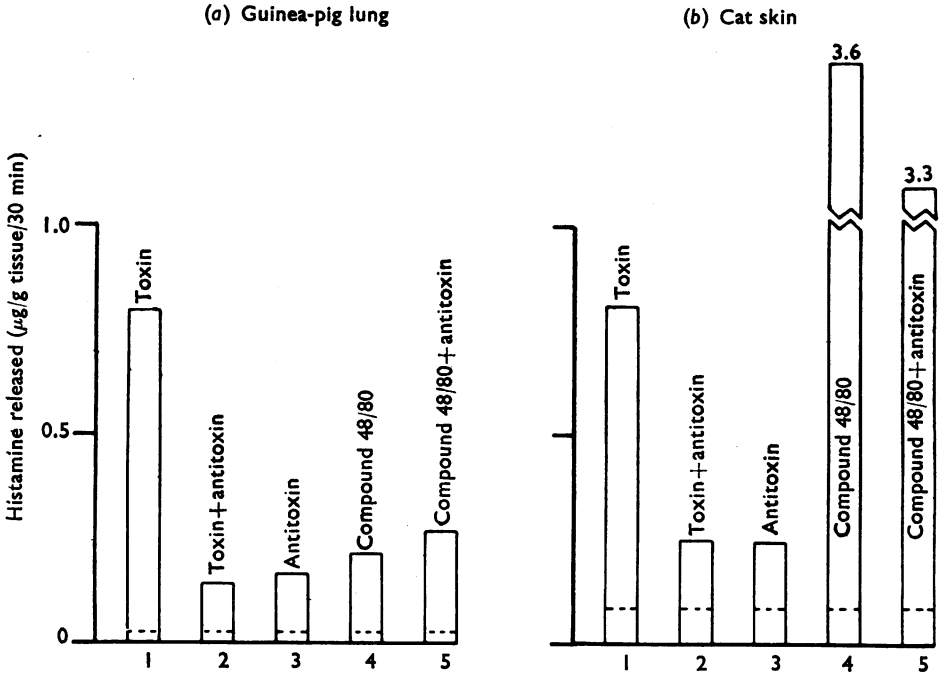

Fig. 4. The effect of staphylococcal anti-α-toxin on the release of histamine by staphylococcal α-toxin and by Compound 48/80 from (*a*) guinea-pig isolated lung and (*b*) cat isolated thigh skin (results from two experiments). Separate portions of each tissue were treated for 90 sec with: (1) 2 H.U./ml. of staphylococcal α-toxin; (2) a mixture of 2 H.U./ml. of α-toxin with 20 I.U./ml. of antitoxin; (3) 20 I.U./ml. of antitoxin alone; (4) Compound 48/80, 1 mg/ml. in (*a*) and 10 μg/ml. in (*b*); or (5) a mixture of Compound 48/80 (concentrations as in (4)) with 20 I.U./ml. of antitoxin. Ordinates give the total amount of histamine (μg/g of tissue) released from each portion of tissue during 30 min incubation after treatment with the agents above. The quantity of histamine released spontaneously during the same period of incubation from a sixth untreated portion of each tissue is indicated by the broken lines.

led to the subsequent release of only a fraction of the amount of histamine released by 2 H.U./ml. of α-toxin alone (column 1) and no more than that released by 20 I.U./ml. of antitoxin alone (column 3). The histamine-releasing action of Compound 48/80 (column 4) was not greatly affected by the admixture of staphylococcal antitoxin solution (column 5). Application of the antitoxin itself appeared to produce a small discharge of histamine (Fig. 4, column 3, compare with the spontaneous release from untreated tissue shown by the broken line). The antitoxin preparation was a pepsin-refined concentrate of horse-serum, a dose of 20 I.U. corresponding to an original volume of 0.6 ml. (approximately) of unrefined serum. Perhaps this action of the antitoxin is a manifestation of the histamine-releasing property of horse serum reported by Feldberg & Schachter (1952).

Release of histamine in the anaesthetized cat

The effect of intravenous injections of staphylococcal α-toxin on the concentration of histamine in the plasma of three anaesthetized cats was studied. In order to obtain

plasma levels approaching the concentration of α-toxin used in *in vitro* experiments with cat isolated skin (2 H.U./ml.), intravenous doses of 100 H.U./kg were given, the plasma volume in the cat being about 47 ml./kg (Altman, Dittmar & Grebe, 1959). In addition, portions of skin were obtained from the thigh of each cat and the amount of histamine released therefrom after application of 2 H.U./ml. of α-toxin for 20 sec was checked. In a fourth cat Compound 48/80 was used in place of α-toxin.

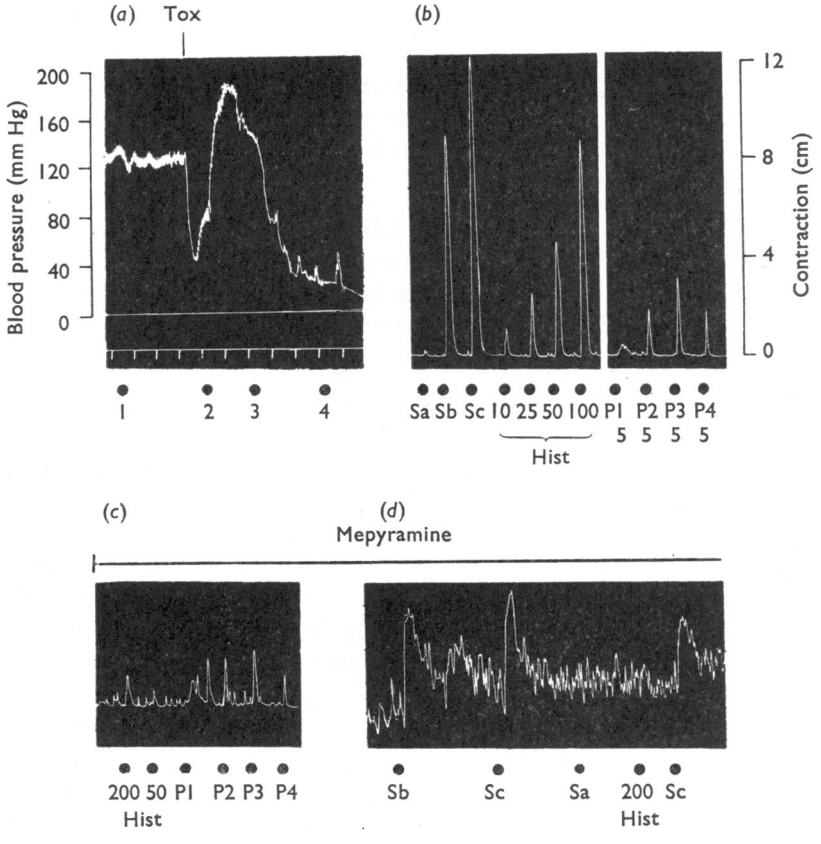

Fig. 5. Effect of staphylococcal α-toxin on (*a*) blood pressure, and (*b*) to (*d*) plasma histamine concentration of an anaesthetized cat. (*a*) Record of carotid arterial blood pressure in a cat (2 kg, anaesthetized with pentobarbitone). At Tox, 100 H.U./kg of staphylococcal α-toxin were injected intravenously. Samples of blood were withdrawn from the femoral artery 3 min before (1) and 1, 3 and 6 min after (2 to 4) the injection of α-toxin. Time marks, 1 min. (*b*) Record of the responses of the superfused guinea-pig ileum to plasma (P1 to P4) obtained from the blood samples 1 to 4; diluted five-times with Tyrode solution. Sa, Sb and Sc are the responses to samples of Tyrode solution in which portions of thigh skin obtained from the cat before injection of α-toxin were incubated for 15 min without treatment (Sa), or after exposure for 20 sec to 0.2 (Sb) or 2 (Sc) H.U./ml. of staphylococcal α-toxin. Concentrations of histamine (Hist) solution are in ng/ml. Records (*c*) and (*d*) show the responses of the ileum after adding 0.2 μg/ml. of mepyramine to the superfusion fiuid. Intervals of 5 and 20 min elapsed between records (*b*) and (*c*), and between (*c*) and (*d*) respectively: during the latter interval spontaneous activity developed in the ileum.

Some results from one experiment using staphylococcal α-toxin are illustrated in Fig. 5. Injection of the α-toxin produced a rapid fall of blood pressure and death within 7 min (Fig. 5,*a*). Similar effects of α-toxin injections were seen in the two other cats, death occurring within 5 and 11 min of injection. Blood collected after the injection was partly haemolysed. Plasma from this blood, diluted 4:1 with Tyrode solution, produced a small contraction of the superfused guinea-pig ileum (Fig. 5,*b*, P2 to P4) which was not seen with plasma (P1) collected before injection of toxin. However, the action of the plasma was not greatly reduced by mepyramine (Fig. 5,*c*) and so could be ascribed only partly, if at all, to the presence of histamine. It was estimated that the concentration of histamine in the plasma did not exceed 0.1 μg/ml. after the injection of α-toxin in each of the three cats tested. This was not due to inadequacies in the technique, because injection of 1 mg/kg of Compound 48/80 into the fourth cat produced a peak plasma histamine concentration of 1.25 μg/ml. 1 min after injection.

Exposure of skin isolated from the cats used in these experiments for 20 sec to 2 H.U./ml. of staphylococcal α-toxin led to the release of 0.8, 1.3 and 1.9 μg of histamine per g of tissue. Treatment of the fourth cat's skin for 20 sec with 20 μg/ml. of Compound 48/80 resulted in the release of 5.1 μg of histamine per g of tissue.

In the experiment illustrated in Fig. 5 the incubation fluid from isolated skin treated with α-toxin produced a " slow contraction " of the superfused guinea-pig ileum in the presence of mepyramine (Fig. 5,*d*). Similar " slow contracting " activity was shown by undiluted incubation fluids from toxin-treated isolated tissues in half the experiments in which the effect of undiluted fluid was tested on guinea-pig ileum in the presence of mepyramine. Plasma from cats injected with α-toxin produced a brief contraction of the superfused ileum in the presence of mepyramine (Fig. 5,*c*). This might have been due to the presence in the plasma of substances derived from lysed erythrocytes. Addition of 0.5 H.U./ml. of staphylococcal α-toxin to whole blood produced similar activity, but no spasmogenic substance was released from cat or guinea-pig plasma by α-toxin.

DISCUSSION

Staphylococcal α-toxin released histamine and, in some experiments, a slow-contracting substance, not antagonized by mepyramine, from guinea-pig isolated lung and cat skin. These findings accord with those of Feldberg & Keogh (1937) and Feldberg & Kellaway (1937), using perfused lungs. Very little histamine was released from other guinea-pig tissues, or from rat tissues, as previously reported by Smith & Miles (1960).

The preparation of α-toxin used in this study was not pure: its method of preparation (described by Brown *et al.*, 1959) does not exclude the presence of other products of staphylococcal metabolism of similar molecular weight to that of the α-toxin. It appears probable that the histamine-releasing action of the preparation was due to its α-toxin content, since it was antagonized by the antitoxin which is devoid of activity against other (β, γ or δ) haemolysins. Antagonism by the antitoxin does not, however, entirely exclude the possibility that nonhaemolytic, antigenic substances of similar molecular weight to the α-toxin might have contributed to the effects of the α-toxin preparation.

The preparation did not show evidence of the staphylokinase activity reported by Lack (1948), since it did not release kinins from plasma proteins. This accords with the finding of Bernheimer & Schwarz (1963) that, in pure form, staphylococcal α-toxin has no proteolytic action.

Intravenous injections of staphylococcal α-toxin did not clearly raise the concentration of histamine in the plasma of anaesthetized cats. Since the α-toxin, in a concentration of 2 H.U./ml., released from the cat isolated skin up to one-third of the amount of histamine released by 20 μg/ml. of Compound 48/80, it might have been anticipated that an intravenous injection of 100 H.U./kg of α-toxin would increase the concentration of histamine in the plasma to a similar fraction of that attained after an injection of 1 mg/kg of Compound 48/80, that is up to about 0.5 μg/ml. Instead the maximum plasma histamine concentration following injections of α-toxin appeared not to exceed 0.1 μg/ml. Several factors might contribute to this unexpectedly low figure. (1) The mepyramine-resistant substance in plasma from intoxicated cats might have diminished the response of the ileum to histamine present in the plasma. (2) The peak rate of histamine release from cat isolated skin following application of α-toxin is rather delayed, and, relative to the total output, is lower than that observed after treament of isolated skin with Compound 48/80 (Fig. 3). There might be a corresponding delay and reduction in the peak plasma histamine level after injection of α-toxin. (3) The direct vasoconstrictor action of the α-toxin (Brown, unpublished) might delay access of the toxin to the skin, or delay penetration of histamine from skin to blood stream. (4) Diffusion of α-toxin from plasma to the tissues through the capillary walls might be restricted by the high molecular weight of the α-toxin (about 44,000, according to Bernheimer & Schwartz, 1963). Irrespective of the reasons, the absence of a marked increase in the concentration of histamine in the plasma of cats following injections of doses of α-toxin sufficiently great to produce death within a few minutes indicates that death must have been due to some action of the α-toxin other than the release of histamine.

The relation between the release of histamine from isolated tissues and the spasmogenic action of the α-toxin is uncertain. Whereas the contractural response can be elicited once only in each piece of tissue, and necessitates application of α-toxin for at least 30 sec (Brown *et al.*, 1959; Wiegershausen, 1962), histamine was released repeatedly from a single piece of tissue, and after exposure times as short as 5 sec. A point of similarity to the spasmogenic action was the presence of a delay of about 90 sec between the application of α-toxin to the tissue and the maximal release of histamine. This delay is unlikely to arise from slow penetration of the toxin in view of the aforementioned ability of the toxin to release histamine after periods of application much shorter than 90 sec. Since the α-toxin did not release appreciable quantities of histamine from the guinea-pig isolated ileum, the local release of histamine is not likely to participate strongly in the contractural response of this tissue to the α-toxin. However, release of histamine from the lungs might contribute to the effects of the α-toxin in guinea-pigs *in vivo*, which have been likened to those of anaphylactic shock (Dworetzky, Zeitlin, Kahn & Baldwin, 1952).

Since the pathogenicity of *Staphylococcus pyogenes* appears to be related closely to α-toxin production (Elek, 1959), it would be interesting to know whether the α-toxin can release histamine from human tissues.

SUMMARY

1. Staphylococcal α-toxin (2 H.U./ml., applied for 90 sec) released histamine from guinea-pig isolated lung and cat skin, but not from other guinea-pig isolated tissues, nor from rat tissues. The amount of histamine released by 2 H.U./ml. of α-toxin was less than that released by 1 mg/ml. of Compound 48/80 with all tissues except for guinea-pig lung.

2. Histamine release by staphylococcal α-toxin was repeatable in the same piece of tissue. The amount released did not vary with different durations of exposure to α-toxin within the range of 5 to 90 sec exposure. There was a delay of about 90 sec between application of α-toxin to cat skin and the maximum release of histamine. This delay was longer than that seen with Compound 48/80. Staphylococcal anti-α-toxin prevented histamine-release by the α-toxin but not by Compound 48/80.

3. Intravenous injection of lethal doses of α-toxin (100 H.U./kg) did not raise the concentration of histamine in the plasma of anaesthetized cats beyond 0.1 μg/ml. Addition of α-toxin to cat or guinea-pig plasma did not result in the formation of spasmogenic substances.

4. It is concluded that histamine-release does not participate strongly in the lethal action of the α-toxin in cats, nor in the spasmogenic action of the α-toxin on the isolated guinea-pig ileum.

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